CHARACTERISATION OF *FUSARIUM* PATHOGENS IN THE UK

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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“Fungi are more closely related to the mycologists that study them than to the plants on which they occur. “

W.F.O. Marasas – *Fusarium* taxonomist

DECLARATION

I declare that the material contained in this thesis is obtained from my own work which has not been submitted to another university for a degree and has not been published previously. Any data, materials and information obtained from external sources, published or unpublished, are identified as such and referenced accordingly.

Viktoria Vágány

ABSTRACT

The primary aim of this project was to identify and characterise *Fusarium* species associated with the basal rot of *Allium* species and internal fruit rot of sweet peppers in the UK. The secondary objective was to develop quick molecular markers to identify *Fusarium oxysporum* f. sp. *cepae* (FOC) causing onion basal rot. Isolates representing diverse *Fusarium* species taken from onions, garlic, shallot and leeks obtained from different production and processing sites in the UK were collected. *F. proliferatum* was found for the first time to be a causal agent of onion basal rot in the UK, but *F. oxysporum* was by far the most common species and *F. oxysporum* isolates belonged to at least two different genotypes based on a sequence comparison of several "housekeeping" genes, and overall, appeared to be polyphyletic. None of the housekeeping genes studied correlate with pathogenicity. Secreted in xylem (*SIX*) genes offer more promise for the specific identification of *F. oxysporum* formae speciales (Lievens *et al*., 2009a) and a homologue of the *SIX7* gene was found only in a few FOC isolates suggesting that *SIX7* is not absolutely necessary for pathogenicity. Whole genome sequencing of a FOC isolate was carried out in order to understand pathogenicity and identify novel effector genes. This work revealed the presence of further homologues of published *SIX* genes, namely *SIX3*, *SIX5* and *SIX9*. The presence of *SIX3* and *SIX5* has only been reported from *F. oxysporum* f. sp. *lycopersici* previously. Additionally, screening of eleven new candidate effector genes suggested that FOC isolates have different gene sets which correspond to the continuous variation of aggressiveness found within the FOC population. *Fusarium lactis, F. proliferatum* and *F. solani* were identified in association with internal fruit rot of sweet pepper obtained from three different production sites in the UK.

LIST OF ABBREVIATIONS

aa	amino acid
AFLP	Amplified fragment length polymorphism
bp	base pair
DNA	Deoxyribonucleic acid
FOC	*Fusarium oxysporum* f. sp. *cepae*
FOL	*Fusarium oxysporum* f. sp. *lycopersici*
f. sp.	Forma specialis (singular)
ff. spp.	Formae speciales (plural)
IGS	Intergenic spacer region
ITS	Internal transcribed spacer region
kb	Kilo base pair
LB	Luria-Bertani medium
l.s.d.	least significant difference
MLST	Multi locus sequence typing
MPC	Mobile pathogenicity chromosome
mtDNA	Mitochondrial DNA
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
RAPD	Random amplified fragment length polymorphism
rDNA	Ribosomal deoxyribonucleic acid
REML	Residual maximum likelihood
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPB2	RNA polymerase II subunit
rRNA	Ribosomal RNA
SCAR	Sequence characterized amplified region
SIX	Secreted in xylem
sp.	Species (singular)
spp.	Species (plural)
SSR	Simple sequence repeat
TEF	Translational elongation factor 1α subunit
VCG	Vegetative compatibility group

CHAPTER 1

GENERAL INTRODUCTION

1.1 *Fusarium* genus

The *Fusarium* genus comprises a wide variety of filamentous fungi recognised as plant, animal and human pathogens and soil saprophytes (Leslie and Summerell, 2006). The members of this genus include the causal agents of some of the most economically devastating plant diseases such as head blight of wheat and Panama disease of bananas (Summerell *et al*., 2010). Some fusaria cause animal and human diseases directly, e.g. keratitis or indirectly by producing mycotoxins such as trichothecenes and fumonisins (Moretti, 2009). Fusaria are widely distributed around the world and can survive for a long time in all types of soils as they are able to utilise a wide range of organic matter (Nelson *et al*., 1981).

Many plant species have at least one *Fusarium*-associated disease and the types of symptoms induced include root or stem rots, cankers, wilts, fruit or seed rots, head blight, and leaf diseases depending on the developmental stages, host plants and *Fusarium* species involved (Moretti, 2009). It is not uncommon for opportunistic species of the genus to colonise plants as a part of a complex of *Fusarium* species. Head blight of cereal crops is caused by several species including, but not limited to, *Fusarium graminearum, F. avenaceum, F. culmorum,* and *F. poae* (Nicholson *et al*., 2003). Head blight of wheat is the most economically devastating plant disease caused by the *Fusarium* species, as wheat is the third most produced cereal after maize and rice in terms of production yield (FAOSTAT, 2010) and it causes severe production losses worldwide, which may be as high as 50 percent (Gilchrist and Dubin, 2002).

F. graminearum, F. proliferatum, F. lactis and several other species can produce a variety of mycotoxins (e.g. trichothecenes and fumonisins) that are

associated with plant disease, as well as with cancer and other growth defects in humans and animals (Masuda *et al*., 2007; Gelderblom *et al*., 1988). Consumption of cereal crops contaminated by trichothecenes can cause chronic and fatal toxicosis in humans and animals through the inhibition of protein synthesis (Rotter *et al*., 1996). Fumonisins are not only capable of increasing the likelihood of cancer, but are hepatotoxic, nephrotoxic and neurotoxic (Stockmann-Juvala and Savolainen, 2008). *F. solani* and *F. oxysporum* species may cause opportunistic infections such as fusariosis, in immunocompromised humans and animals (O'Donnell *et al*., 2004). Ocular infections, keratitis, by *F. solani and F. oxysporum* have also been observed recently among contact lens users (Chang *et al.,* 2006).

Many fusaria serve a beneficial function in soil habitats as saprophytes degrading plant debris or preventing plant diseases (Edel *et al*., 1997). *Fusarium oxysporum, F. solani, F. culmorum* and *F. redolens* are frequently isolated from soil (Balmas *et al*., 2010).

Additionally, some species may be used for human nourishment; *Fusarium veneatum* myco-protein, for example, is produced industrially and marketed as Quorn (Wiebe, 2002). Furthermore, *F. oxysporum* was used for the synthesis of silver nanoparticles (Ahmad *et al*., 2003).

1.2 Classification of *Fusarium* genus

The *Fusarium* genus was introduced by Link in 1809 and the name refers to the characteristic spindle-shaped (Latin *fusus* means spindle) macroconidia. The genus *Fusarium* (anamorph) belongs to the *Ascomycota* phylum, *Sordariomycetes* class, *Hypocreales* order, and is also known by its teleomorphs *Gibberella* and *Nectria* (Leslie and Summerell, 2006). During the last 200 years the number of

Fusarium species has varied from 5 to 1000 based on the taxonomical systems used; at the moment more than 80 *Fusarium* species are recognised (Leslie & Summerell, 2006).

Discrimination of *Fusarium* species was traditionally based on morphological characters (morphological species recognition) that enabled the identification of some of the most important pathogens (Moretti, 2009). However, the genus lacks reliable morphological characters (Geiser *et al.*, 2004), and it is therefore very difficult for researchers with little experience to characterise *Fusarium* species based purely on morphological traits. For example, differences in the shape and size of the macroconidia and microconidia can be subjective and depend upon the environment in which they are produced (Leslie and Summerell, 2006).

Fusarium species can be recognized based on their ability to actually or potentially interbreed with other members of the population (biological species recognition), rather than their morphological appearance (Mayr, 1942). For instance, the *Gibberella fujikuroi* species complex was divided into at least eleven different biological species or mating populations (Britz *et al*., 1999; Klaasen and Nelson, 1996; Leslie, 1991; Zeller *et al*., 2003; Geiser *et al*., 2005). On the other hand, it is difficult to apply the concept of biological species to fusaria because some lacks sexual reproduction (Kerenyi *et al*., 1999). Although several *Fusarium* species, i.e. *F. solani* and *F. graminearum*, mate in either a homothallic or heterothallic manner, important pathogenic species, including *F. avenaceum*, *F. culmorum* and *F. equiseti*, have no known sexual stage.

Phylogenetic analyses (phylogenetic species recognition) can be useful to resolve species level identification of fungi without a known sexual stage. Many phylogenetic studies use DNA sequences of one or two loci of a few isolates, which

can be misleading. For example, the ribosomal internally transcribed spacer (ITS) can have non-orthologous sequences of the ITS2 region in individual representatives of the *Gibberella* clade (O`Donnell and Cigelnik, 1997). Because of these issues, the most widely accepted concept among mycologists and fungal biologists for *Fusarium* and other fungal species boundaries is a phylogenetic approach, based on the concordance of multiple gene genealogies when compared with morphology and reproductive behaviour (Taylor *et al*., 2000). For example, O'Donnell and co-workers used nuclear 28S rDNA, mitochondrial small subunit of ribosomal rDNA (mtSSU), and β-tubulin gene sequences to resolve species boundaries within the *Gibberella fujikuroi* species complex (O'Donnell *et al*., 1998b). In this study, 46 phylogenetic species were predicted, eleven of which were previously identified based on biological species recognition, but 23 of which were proposed to be new species. This shows that phylogenetics can be successfully applied to species identification and also helps the recognition of cryptic species. Another example is the *F. graminearum* clade where phylogenetic species recognition revealed the presence of nine phylogenetic species that were previously treated as one species (O'Donnell *et al*., 2004; O'Donnell *et al*., 2000).

1.3 Sub-specific ranks to characterise *Fusarium* species

Many sub-specific ranks are currently in use for the *Fusarium* species, such as forma specialis, including race and vegetative compatibility groups. Forma specialis (f. sp.) for short, and the plural formae speciales (ff. spp.) is the most commonly used sub-specific rank among *Fusarium* species and refers to the host preference of isolates that are morphologically indistinguishable. For example, *F.*

solani f. sp. *pisi* causes wilting of peas while *F. solani* f. sp. *phaseoli* attacks beans. It has to be noted that formae speciales have no nomenclatural standing and are not governed by the International Code of Botanical Nomenclature as pathogenicity is not a reliable ancestral trait (Greuter *et al.* 2000). Each f. sp. can also be further subdivided into physiological races and vegetative compatibility groups (VCGs).

Races refer to the host cultivar preference of a given *forma speciales* (f. sp.). A classical gene-for-gene interaction was proposed as the basis for the development of host cultivars resistant to *Fusarium* diseases (Takken and Rep, 2010). Vegetative compatibility between isolates refers to the fusion of hyphae that anastomose to form a stable heterokaryon (Puhalla, 1985). Isolates that can form a stable heterokaryon are compatible and therefore can be categorised in the same vegetative compatibility group (VCG). The VCG phenotype has a multigenic basis in *Fusarium* (approximately 10-15 *vic* loci) and can be used to identify a set of isolates that share common alleles at those loci (Leslie and Summerell, 2006). If the alleles of one (or more) *vic* loci differ, then the heterokaryon is unstable which leads to cell lysis after hyphal fusion (Leslie, 1993). In practice, complementary nitrate-reductase mutants are used to determine vegetative compatibility of *Fusarium* isolates while growing on nitrate-containing media (Puhalla, 1985).

1.4 *Fusarium* pathogens in the UK

There are several economically important plant diseases caused by members of the *Fusarium* genus in UK cropping-systems. Fusarium head blight (FHB) of wheat is caused by co-existing *F. graminearum, F. culmorum, F. avenaceum, F. poae,* and *F. langsethiae* species and is a re-emerging threat to British crop

production with an increase in disease incidence to 39% over the last 10 years in the UK (Brown *et al*., 2011; Nicholson *et al*., 2003; CropMonitor). Also, toxin producers like *F. culmorum* and *F. graminearum* have been more frequently isolated from wheat compared to toxin non-producing competitor species such as *Microdochium nivale* (formerly *Fusarium nivale*) (Gosman *et al*., 2007).

Potato is also affected by *Fusarium* pathogens in the UK. Dry rot of potato is caused by *F. coeruleum, F. avenaceum, F. culmorum* and *F. sambucinum* and affects at least 1% of British potato tubers (Peters *et al*., 2008). Additionally, asparagus can suffer from asparagus decline, a disease associated with *F. oxysporum* f. sp. *asparagi*, *F. proliferatum*, *F. solani* and *F. redolens* (Wong and Jeffries, 2006). Legume hosts, such as peas and beans, are suffering from foot-rot symptoms caused by *F. solani* (Clarkson, 1978).

Important *Allium* crops such as onion, shallot, garlic, and leek are all affected by *F. oxysporum*. Onion basal rot, caused by *F. oxysporum* f. sp. *cepae,* was first reported in 1980 in Essex, UK and since 2006, onion basal rot is an increasing problem to UK growers (A. Findlay, BOPA, pers. comm.). Ornamental crops such as daffodils can suffer from basal rot symptoms caused by *F. oxysporum* f. sp. *narcissi* in the UK (Hanks and Carder, 2003). Another recent emerging *Fusarium* disease is internal pepper fruit rot, which could cause up to 37% loss to crops in the UK (O'Neill, 2008).

1.5 Molecular identification of the *Fusarium* species

A community effort is being made to enable the accurate identification of *Fusarium* isolates based on sequence similarity to well-characterised *Fusarium*

species (Geiser *et al*., 2004). A multiple sequence database called Fusarium-ID contains information on more than 1800 isolates representing 76 *Fusarium* species (Park *et al*., 2011; O'Donnell *et al*., 2010). New isolates can be initially identified based on sequences for the translation elongation factor 1α (*TEF*) gene, as well as two genes encoding the largest and second largest subunits of RNA polymerase (*RPB1* and *RPB2*, respectively) sequences against the Fusarium-ID sequence database. These three genes are highly informative at the species level in *Fusarium*; non-orthologous copies of the genes have not been detected in the genus and conserved primers are available that can be used to amplify and sequence these genes in all fusaria described to date (O'Donnell *et al*., 1998a; O'Donnell *et al*., 2010). More precise identification of new *Fusarium* isolates may require phylogenetic analysis based on multiple markers, e.g. the query sequence may represent a novel species not represented in Fusarium-ID. Sequence data of several other markers such as phosphate permease genes (*PHO*), β-tubulin (*TUB*), calmodulin (*CAL*), UTP-ammonia ligase (*URA*), trichothecene 3-O-acetyltransferase (*TRI101*), a putative reductase (*RED*), histone 3 (*H3*), intergenic spacer (*IGS*), the small nuclear subunit of ribosomal rDNA (*18S-rDNA*), the large nuclear subunit of ribosomal rDNA (*28S-rDNA*), the small mitochondrial subunit of ribosomal rDNA (*mtSSU*), and mating type genes (*MAT1-1* and *MAT1-2*) are also available for a multi locus characterization of *Fusarium* pathogens and their populations (Park *et al*., 2011).

1.6 *Fusarium oxysporum* species complex

Fusarium oxysporum Schlecht., is the most common species of the genus, commonly occurs in the soil as a saprophyte but is also a well-known plant endophyte and an emerging pathogen of humans. It was recently identified as the 5th

most important plant pathogenic fungus based on its economic and scientific impact after *Magnaporthe oryzae*, *Botrytis cinerea*, *Puccinia* spp. and *Fusarium graminearum* (Dean *et al.*, 2012). One of the most devastating diseases caused by *F. oxysporum* is Panama disease of banana that almost completely destroyed the export trade in the 1960s (Ploetz, 2000). *F. oxysporum* can cause disease losses in almost all vegetable and ornamental crops as well as field crops such as cotton and a few palm species (Di Pietro *et al*., 2003).

F. oxysporum as a species was defined by Snyder and Hansen over 50 years ago but more recent work indicates this taxon is actually a genetically heterogeneous polytypic morphospecies (Kistler, 2001). No sexual stage has been identified for *F. oxysporum* and therefore it is regarded as a species complex; a complex of clonal lineages within the *Fusarium* genus. Despite the predominant asexual reproduction of *F. oxysporum,* mating type loci were identified and the associated *MAT* genes were found to be functioning correctly (Yun *et al*., 2000).

The *F. oxysporum* species complex comprises different formae speciales that collectively cause wilts of leaves and stem, root, bulb and crown rots as well as both pre- and post-emergence damping-off of over 100 different plant host species. It has been shown that four economically important formae speciales: *cubense, lycopersici, radicis-lycopersici* and *melonis* were found to be polyphyletic (*i.e.* several independent clonal lineages are present in each f. sp.) based on nuclear and mitochondrial gene genealogies (O'Donnell *et al.*, 1998a; Figure 1).

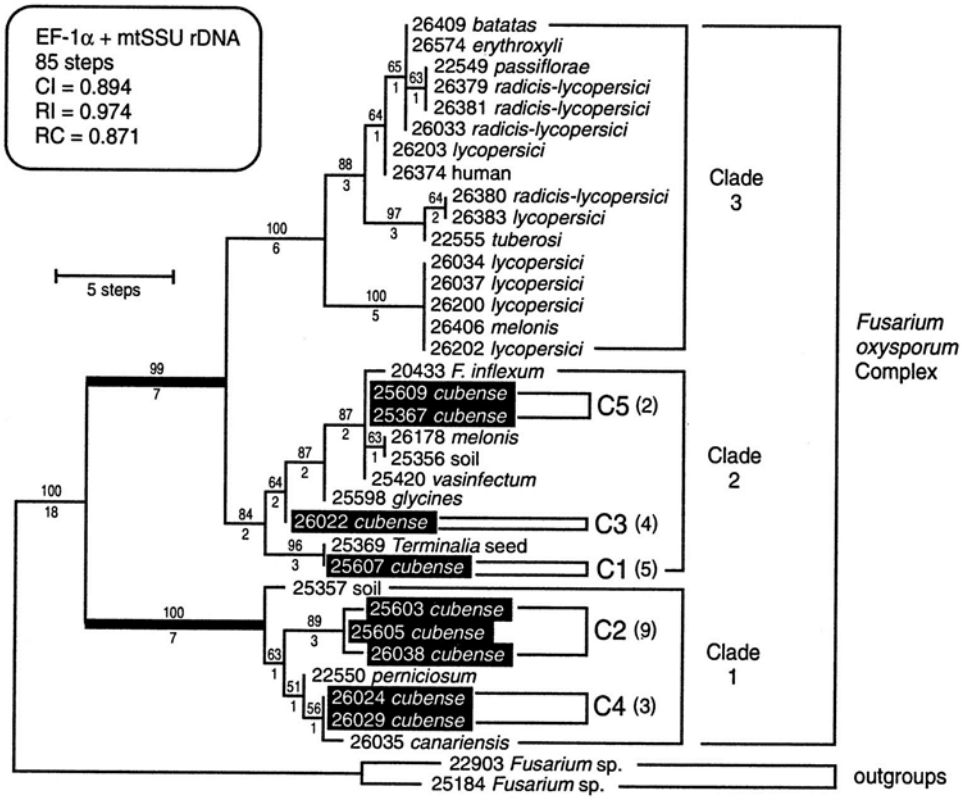


Figure 1. Single most-parsimonious phylogram by parsimony branch-and-bound search implemented in PAUP based on the combined translation elongation factor 1α (*TEF*) gene and mtSSU rDNA dataset for the *Fusarium oxysporum* complex rooted with sequences from *Fusarium* sp. NRRL 22903 and 25184. The five clonal lineages (C1-C5) of ‘‘*F. oxysporum* f. sp. *cubense*’’ are numbered according to the frequency with which each has been recovered from diseased bananas (after O’Donnell *et al*., 1998a).

Furthermore, amplified fragment length polymorphism (AFLP) markers were used in an extensive study by Baayen *et al.* (2000) to identify monophyletic and polyphyletic/paraphyletic formae speciales. This study also revealed that formae speciales comprising more than one VCG are not usually monophyletic (e.g. *lycopersici, gladioli, asparagi, lini,* and *dianthi*), but isolates originating from different host species can be grouped in the same clade. This finding suggests that pathogenic ability emerged convergently as no sexual stage has been observed in this fungus. Moreover, vegetative hyphal fusion (anastomosis) can only occur between

compatible *F. oxysporum* isolates (vegetative compatibility) which together results in the development of genetically isolated clonal lines. However, there are two studies published which provide some explanation: First, parasexual recombination between vegetatively incompatible strains has been observed *in vitro* which resulted in stable hybrids (Molnar *et al.*, 1990; Teunissen *et al*., 2002). Second, mobile pathogenicity chromosomes (MPCs) exist in *F. oxysporum* f. sp. *lycopersici* (FOL), the forma specialis affecting tomato, and the transfer of two of these MPCs between isolates of *F. oxysporum* converted a non-pathogenic isolate into a pathogenic one, suggesting that these chromosomes can spread among clonal lineages through horizontal gene transfer (Ma *et al*., 2010).

1.7 Disease cycle and infection process of *Fusarium oxysporum*

Fusarium oxysporum is a soil-borne pathogen of a broad range of agricultural and ornamental crops and also a common saprophyte on plant debris in soil. In soil it can survive as mycelium on decaying plant debris or as asexual micro- and macrospores or chlamydospores (Abawi and Lorbeer, 1972). Plants are infected by directed growth of *F. oxysporum* conidial germ tubes or mycelium which penetrates the root epidermis at the root tips or at natural wounds such as during the formation of lateral roots (Cooper and Bishop, 1983ab; Hutson and Smith, 1983). The infectious hypha infects the root through natural openings between epidermal root cells and grows through the root cortex intercellularly until it reaches the xylem's pits. In the xylem vessels, the mycelium branches produce chlamydospores, which are carried by the plant's sap stream (van der Does *et al*., 2008; Michielse and Rep, 2009). The colonisation of the xylem leads to the blockage of the xylem vessels and

/or toxin production and host defence responses, including production of pectic gels, gums and tyloses and vessel crushing by the proliferation of parenchyma cells (Beckman, 1987); and causing various symptoms such as wilting, chlorosis, necrosis, leaf drop, browning of the vascular system, and stunting.

Resistant tomato plants respond quicker to *Fusarium oxysporum* f. sp. *lycopersici* and contain the fungus by callose deposition and production of pectic gels, tyloses and gums in the infected xylem vessels instead of a classical hypersensitive response (HR) (i.e. cell death) than those that are susceptible (Mes *et al*., 2000).

F. oxysporum can spread short distances by irrigation water, contaminated agricultural machinery, via air or long distances by infected seeds and planting material (Abawi and Lorbeer, 1972).

1.8 Molecular mechanisms in the pathogenicity of *Fusarium oxysporum*

Several genes which are important in molecular mechanisms underpinning the pathogenicity and virulence of *F. oxysporum* have been identified during the last ten years. Forward and reverse genetic approaches have been applied to understand the importance of genes involved in signal transduction, utilisation of host nutrients, transcription and defence mechanisms.

1.8.1 Effectors

Effectors are “secreted proteins and other molecules which allow plant-associated organisms to modulate plant defence circuitry and enable colonization of plant tissue” (Hogenhout *et al*, 2009).

Plants have developed two lines of defence against pathogens. The first one provides defence against all pathogens and is based on the recognition of pathogen-associated molecular patterns (PAMPs or MAMPs for microbe-associated molecular patterns) such as chitin and β-glucan (main components of the fungal cell wall). This is termed PAMP-triggered immunity (PTI; Zipfel and Rathjen, 2008). Pathogens have developed ways to overcome basal defence using effectors, and as a response to this, plants developed a second line of defence to recognise these effectors. This is known as effector-triggered immunity (ETI; de Wit *et al*., 2009).

Small *in planta* secreted effectors called “secreted in xylem” (Six) proteins have been identified from FOL which interact (directly or indirectly) with tomato resistance genes (Takken and Rep, 2010). Several dominant plant resistance (*I*, for immunity) genes against different FOL races were identified (Huang and Lindhout, 1997): *I* and *I-1* genes confer resistance only to FOL race 1; *I*-2 is effective against FOL race 2 (which overcomes *I* and *I-1*); and I-3 confers resistance to FOL race 3 (and overcomes *I*, *I*-1 and *I*-2) (Rep *et al.*, 2005). Tomato plants carrying resistance (*I*, *I-1*, *I-2* and *I-3*) genes are able to recognise Six1, Six3 and Six4 proteins produced by the fungus, resulting in the tomato becoming resistant to the disease. The recognition of Six1, Six3 and Six4 proteins by the tomato leads to avirulence, and therefore the encoding genes are called avirulence genes (*AVR1- AVR3*; Rep *et al*., 2004).

Gene knockout experiments showed that Six4 (Avr1) is required for *I* and *I-1*-mediated disease resistance, whereas Six3 (Avr2) is needed for *I-2*-mediated resistance and Six1 (Avr3) is essential for both I-3-mediated and I-2-mediated resistance (Houterman *et al*., 2008; Houterman *et al*., 2009). The *SIX4* gene is only present in FOL race 1 strains while race 2 and race 3 isolates differ only in their *SIX3* gene sequences (Lievens *et al*, 2009a). Therefore it is likely that race 2 strains evolved through loss of *SIX4* (*AVR1*) from race 1 strains, while mutations in the *SIX3* (*AVR2*) led to the development of race 3 strains (Houterman *et al*., 2009).

Comparison of genome sequences of FOL, *F. graminearum*, and *F. verticillioides* revealed mobile pathogenicity chromosomes (MPCs) in FOL (Ma *et al*., 2010). Genomic regions were found in FOL that include four entire chromosomes (chromosomes 3, 6, 14 and 15) and parts of chromosome 1 and 2 which are rich in transposons and genes related to pathogenicity. Interestingly, *SIX4* (*AVR1*), *SIX3* (*AVR2*) and *SIX1* (*AVR3*) are located on chromosome 14 along with *SIX2, SIX5, SIX6* and *SIX7*. The transfer of chromosome 14 between isolates of *F. oxysporum* converted a non-pathogenic isolate into a pathogen (Ma *et al*, 2010).

To date only one resistance gene (*I-2*) has been cloned from the tomato which confers resistance to FOL race 2 strains (Simons *at al*., 1998). *I-2* locus is encoding a protein containing a nucleotide binding site motif and leucine-rich repeats (Simons *et al*., 1998). Expression studies revealed that *I-2* is abundant in the tissue surrounding the xylem vessels in the roots and stem of tomato plants (Mes *et al*., 2000). Avr2 recognition by I-2 can be artificially induced in leaves and stems, and is visible as a hypersensitive response (Houterman *et al*., 2009). Amino acid mutations (V41 → M, R45 → H or R46 → P) of Avr2 prevent recognition by I-2 in tomato, but do not interrupt its active site (Houterman *et al*., 2009). It is also known that Avr2 is

secreted into the xylem, but I-2 is present in the tomato cytosol (Houterman *et al*., 2009).

Thus far it has not been shown how Avr2 and I-2 and interact, although three possibilities were proposed by Takken and Rep (2010): (i) direct interaction of the two proteins, (ii) I-2 guards the target of Avr2 (guard model) or (iii) I-2 mimics the target of Avr2 (decoy model). No evidence has been found for the direct interaction of the proteins so far (Takken and Rep, 2010). Three proteins were found binding to Avr2 which were identified as chaperones and other proteins involved in protein maturation (van Bentem *et al*., 2005; Takken and Rep, 2010). Two additional proteins were identified from the tomato as interactors of *I-2*, which when stably silenced did not compromise *I-2*-mediated disease resistance (Lukasik-Shreepaathy *et al*., 2012).

The molecular basis of *Fusarium*-tomato interactions can be summarized as an example of zig-zag evolution of plant-pathogen interactions (Jones & Dangl, 2006; Takken and Rep, 2010). In phase 1, soil habitant *F. oxysporum* is recognized by tomato, but unable to invade plant, due to PTI. In phase 2, *F. oxysporum* deploys the combination of effectors, such as Avr2 and Avr3, enzymes and metabolites to overcome basal immunity. In phase 3, Avr2 and Avr3 directly or indirectly are recognised by nucleotide-binding and leucine-rich repeat (NB-LRR) proteins I-2 and I-3, respectively, that trigger ETI. In phase 4, FOL escaped recognition by point mutations in Avr2 and also by suppressing both I-2 and I-3 by Avr1. In phase 5, in tomato I-1 evolved to recognize Avr1 to prevent infection by FOL.

1.8.2 Transcription factors

Two transcription factors required for *F. oxysporum* pathogenicity (*FOW2* and *SGE1*) and two others (*FTF1* and *FOST12*) present only in highly virulent isolates have been identified (Michielse *et al.*, 2009b; Imazaki *et al*., 2007; Ramos *et al*., 2007; Asuncion *et al*., 2010). *SGE1* (*SIX* Gene Expression 1), a nuclear protein, is essential for pathogenic growth and the expression of *SIX* genes of FOL, but, it is not required for root colonisation and penetration (Michielse *et al*, 2009b). The only difference between the Sge1 mutant and wild type isolates, apart from pathogenicity, was reduced conidiation.

Another important regulator of pathogenic development is the Fow2 of *F. oxysporum* f. sp. *melonis* (the *F. oxysporum* gene required for wilt symptom 2), a Zn(II)2Cys6-type transcription regulator, that is required to invade roots and colonise melon tissue, but does not impair vegetative growth and conidiation (Imazaki *et al*., 2007).

A third transcription factor, *Fusarium* transcription factor 1 (*FTF1*), also has a Zn(II)2-Cys6 DNA-binding motif and plays a role in the establishment of the fungus within the plant and/or the progress of the disease (Ramos *et al*., 2007). *FTF1* is present as multiple copies in highly virulent *F. oxysporum* f. sp. *phaseoli* strains, while weakly virulent strains lack *FTF1*. Moreover, it is highly expressed during the first stages of common bean colonisation and the expression of *FTF1* correlates with the degree of virulence (de Vega-Bartol *et al*., 2011).

A fourth transcription factor (Fost12) of *F. oxysporum* f. sp. *phaseoli*, a homologue of Ste12 of *Saccharomyces cerevisiae*, is highly expressed during the period between 12 and 24 h after inoculation of common bean plants. Disruption mutants showed significant reduction in virulence and the transcriptional analyses of

FOST12 in several *F. oxysporum* f. sp. *phaseoli* strains during axenic growth suggest that Fost12 is a virulence factor required to deal with the nitrogen stress confronted by the pathogen during host plant colonisation (Asuncion *et al*., 2010).

1.8.3 Detoxification of antifungal compounds

In response to pathogen invasion, plants produce a great variety of soluble and cell-wall-bound phenolic compounds (such as ferulic, vanillin and coumaric acid) which can inhibit fungal growth and / or by their accumulation (lignification) reinforce the plant cell-wall (Niemann *et al*., 1991). In addition to this, some plants produce saponins (e.g. tomatine produced by tomato) or pterocarpans (e.g. pisatin produced by peas) to inhibit fungal infection. Several fungal species, including *F. oxysporum*, produce enzymes to degrade these antifungal compounds to overcome plant defence.

Tomatinase and tomatine hydrolase, have been discovered from FOL, *Verticillium albo-atrum*, and *Botrytis cinerea, F. solani* (Lairini and Ruiz-Rubio, 1998; Sandrock and VanEtten, 1998; Quidde *et al*., 1998; Roldan-Arjona *et al*., 1999). Five putative tomatinase genes present in the genome of FOL, and the disruption of one (*TOM1*) of them resulted in 25% loss in tomatinase activity and a delay in the progression of the disease symptoms (Pareja-Jaime *et al*., 2008). On the other hand, the overexpression of *TOM1* accelerated the spread of the symptoms.

Pisatin-demethylase is involved in the detoxification of pisatin produced by pea plants and was originally found in *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*) (VanEtten *et al*., 1980). Recently, its functioning homologue (*FoPDA1*) was discovered in *Fusarium oxysporum* f. sp. *pisi*. The transformation of

the *FoPDA1* gene into *Fusarium oxysporum* f. sp. *lini* promoted pathogenicity towards peas (Coleman *et al*., 2011ab).

Phenolic compounds can be degraded either through the β-ketoadipate pathway or by laccases (Michielse *et al*., 2012; Canero and Roncero, 2008). The deletion of 3-carboxy-*cis,cis*-muconate lactonizing enzyme (*CMLE*) of FOL, an enzyme of the β-ketoadipate pathway, led to increased sensitivity to phenolic compounds and impaired root invasion and therefore loss of pathogenicity (Michielse *et al*., 2012). The molecular and functional characterisation of six putative laccase genes of FOL (*LCC1*- to *LCC5*, and *LCC9*) revealed that *LCC1, LCC3*, and *LCC9* are expressed during root and stem infection, although targeted inactivation of *LCC1, LCC3*, and *LCC5* did not have any effect on virulence (Canero and Roncero, 2008). Inactivation of a chloride channel coding gene (*CLC1*) caused a deficiency in laccase activity (probably by disabling the insertion of copper cofactor into maturing laccases) which was more severe than that found in any of the single laccase mutants (Canero and Roncero, 2008). This suggests that laccases may be collectively required for pathogenicity.

1.8.4 Enzymatic degradation of the plant cell wall

A plethora of plant cell wall degrading enzymes (CDWEs) have been predicted to play a role during infection of *F. oxysporum* as they are produced during host penetration and colonisation (Di Pietro *et al*., 2003). Individual inactivation of pectate lyases (*PL1*), xylanases (*XYL3, XYL4* and *XTL5*), proteases (*PRT1*) and polygalacturonases (*PG1, PG5* and *PGX4*) did not have an effect on the virulence of *F. oxysporum* (Di Pietro and Roncero, 1998; Huertas-Gonzalez *et al*., 1999ab; Garcia-Maceira *et al*., 2000; Garcia-Maceira *et al*., 2001; Di Pietro *et al*., 2001b;

Gomez-Gomez *et al*., 2001; Gomez-Gomez *et al*., 2002). Inactivation of a transcription factor (*XLNR*) that activates the expression of xylanases and cellulases reduced the xylanase activity, but did not have an effect on virulence (Calero-Nieto *et al*., 2007). Similarly, deletion of a transcription factor (*CTF1*) that activates cutinase and lipase genes was found to be dispensable for the virulence of *F. oxysporum* (Rocha *et al*., 2008). Cell-wall degrading enzymes are under carbon catabolite repression, which is controlled by a sucrose non-fermenting 1 gene (*SNF1*). The disruption of this gene reduced expression of several genes encoding cell wall-degrading enzymes and delayed infection of the host (Ospina-Giraldo *et al*., 2003). An F-box protein (*FRP1*) is involved in the expression of CDWEs, non-sugar carbon catabolism, host colonisation and therefore pathogenicity (Duyvesteijn *et al*., 2005; Jonkers *et al*., 2009; Jonkers *et al*., 2011).

These results suggests that enzymes involved in plant cell wall degradation are important virulence factors in *F. oxysporum*, although multiple copies of functionally redundant enzymes can mask the loss of individual CDWEs.

1.8.5 Signal transduction

Two conserved signal transduction cascades are present in *F. oxysporum*, a cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) cascade and a mitogen-activated protein kinase (MAPK) cascade. These are required in order to recognise the host, attach to and penetrate the root epidermis, overcome the plant immune system, secrete toxins and small peptides and sporulate (Di Pietro *et al*., 2003).

FOL isolates were unable to attach to tomato roots and grow invasively in fruit tissue when MAPK (*FMK1*) was inactivated, although this mutant showed

normal growth (except becoming hydrophilic) and sporulation on artificial media. These mutants produced less polygalacturonase and pectate lyase compared to wild type which suggests that Fmk1 controls the expression of enzymes during early stages of infection (Di Pietro *et al.*, 2001a). Recently a mucin-type transmembrane sensor (Msb2) was discovered that promotes invasive growth and plant infection upstream of Fmk1 while contributing to cell integrity through a distinct pathway (Pérez-Nadales and Di Pietro, 2011).

Other proteins involved in the MAPK pathway are the heterotrimeric G-proteins (consisting of α-,β- and γ-subunits), which play a role in the regulation of hyphal growth, sporulation and virulence. The ortholog of the Gαi-protein coding gene in *F. oxysporum* f. sp. *cucumerinum*, *FGA1*, was disrupted which led to reduced conidiation, virulence and also cAMP level (Jain *et al*., 2002). This decrease in the cAMP level might suggest that Fga1 is involved in the activation of cAMP-PKA cascade (Jain *et al*., 2002). Another Gαi-protein coding gene in *F. oxysporum* f. sp. *cucumerinum*, *FGA2* was identified and the deletion of this gene led to complete loss of virulence and increased heat-tolerance, but did not have an effect on colony morphology (Jain *et al*., 2005). Disruption of the Gβ subunit in *F. oxysporum* f. sp. *cucumerinum* resulted in reduced virulence on cucumber and a decrease in conidiation and cAMP level (Jain *et al*., 2003). These results were confirmed by using a FOL strain and additional observations were made, e.g. *FGB1* mutants did not form hyphal branches in liquid media (Delgado-Jarana *et al*., 2005). Deletion mutants of cyclic AMP-dependent protein kinase A of *F. oxysporum* (FoCPKA) showed reduced growth, morphology, root attachment, penetration, and pathogenesis in *Arabidopsis thaliana* (Kim *et al.*, 2011). A striking finding is that MAPK (*FMK1*) is dispensable for virulence in immunocompromised mice (*Mus musculus*) and wax

moths (*Galleria mellonella*) (Di Pietro *et al*., 2001a; Ortoneda *et al*., 2004; Navarro-Velasco *et al.*, 2011). All these results imply that signal transduction has a key role during infection to respond to host defence mechanisms and to adapt to plant and animal hosts.

1.8.6 Response to ambient pH

An important environmental factor that influences fungal growth and development is ambient pH. In *F. oxysporum*, a pH responsive transcription factor (PacC) that binds the consensus 5'-GCCAAG-3' sequence was found essential for virulence in immunocompromised mice and wax moths, but not in tomatoes (Ortoneda *et al*., 2004; Navarro-Velasco *et al.*, 2011). Inactivation of PacC, which is an activator of genes expressed in alkaline conditions and a repressor of those expressed in acidic conditions, induced increased virulence towards tomatoes, while its over expression led to reduced virulence (Caracuel *et al*., 2003).

1.8.7 Fungal cell wall maintenance

Chitin synthesis has been shown to be crucial for pathogenicity and hence several chitin synthase gene knock-out mutants on *F. oxysporum* lost virulence (*CHV5b*) or had reduced virulence (*CHV5, CHV7, CHV2*) (Martin-Urdiroz *et al*., 2008; Madrid *et al*., 2003). Additional enzymes, such as β-1,3-glucanosyltransferases (Gas1) and GTPase (Rho1), play active roles in fungal cell wall biosynthesis. Rho is a small monomeric G protein that functions as an essential signalling component of the fungal cell wall–integrity pathway (Caracuel *et al*., 2005). Inactivation of Gas1

dramatically reduced virulence on the tomato, exhibited restricted colony growth on solid substrates and increased the expression of *CHSV* (Caracuel *et al*., 2005).

1.8.8 Response to nitrogen source

Fungi can utilise a wide range of nitrogen sources, but ammonia and glutamine are preferred, and when these are not available, for example during *in planta* growth, other secondary sources can be used (Marzluf, 1997). Utilisation of these sources is highly regulated and requires *de novo* synthesis of catabolic enzymes and permeases. A key component in nitrogen utilisation is the global nitrogen regulator, a transcription factor (Marzluf, 1997). Disruption of this nitrogen regulator in *F. oxysporum* (FNR1) eliminated the expression of nutrition genes normally induced during the early phase of infection (Divon *et al*., 2006). Fmk1 MAPK of *F. oxysporum* controls virulence functions such as invasive growth, vegetative hyphal fusion and host adhesion, which are strongly repressed in the presence of the preferred nitrogen source ammonium (López-Berges *et al*., 2010).

1.8.9 Additional genes involved in pathogenicity

A mitochondrial carrier protein (Fow1) was found to be specifically required for pathogenicity in *F. oxysporum* f. sp. *melonis*. Deletion mutants of *FOW1* showed normal growth and conidiation in culture, but they were unable to colonise plant tissue and therefore had reduced virulence (Inoue *et al*., 2002).

Disruption of argininosuccinate lyase (*ARG1*) of *F. oxysporum*, catalysing the last step for arginine biosynthesis, led to reduced virulence towards melon (Namiki *et*

al., 2001). Recently, peroxisomal function was found to be necessary for the pathogenesis of *F. oxysporum* (Michielse *et al*., 2009a). Peroxisomes, organelles in filamentous fungi, are involved in the β-oxidation of fatty acids, peroxide detoxification and plugging the septal pores after hyphal wounding. Deletion of two genes involved in the biogenesis of peroxisomes (*PEX12* and *PEX26*) caused loss of virulence and reduced growth on fatty acids (Michielse *et al*., 2009a).

Moreover, genes (*FOXG_08602*) similar to spherullin (involved in tissue desiccation or hydration), transcriptional regulators (*FOXG_03318*) and two proteins with unknown function (*FOXG_09487* and *FOXG_02054*) were proven to be important for the pathogenesis of FOL (Michielse *et al*., 2009a).

1.9 Characteristics of fungal effectors

Host specificity may be determined by effector genes, therefore can potentially be used for the detection and identification of plant pathogens. Known effector genes have at least one of the characteristics which are listed in this section. Therefore, potential effector genes can be identified based on these criteria. This section is predominantly focussed on fungal and oomycete effector genes. Characteristics of *F. oxysporum* effectors are discussed in Chapter 4.

1.9.1 Trigger or prevention of host defence responses

Some effectors trigger host defence responses, whilst others prevent them depending on the repertoire of resistance genes which are present in the host. There are few examples available where it is known which host proteins are targeted. Avr2 of *Cladosporium fulvum* inhibits the hypersensitive response by binding to the

extracellular tomato cysteine protease Rcr3 (Rooney *et al*., 2005). Another effector of *C. fulvum* is Avr4 which binds to plant chitinases to prevent fungal cell degradation (van den Burg *et al.*, 2006). Effectors not only exist in plant pathogens, but also in the mutualistic ectomycorrhizal basidiomycete *Laccaria bicolor* and in the arbuscular mycorrhizal fungus *Glomus intraradices* promoting symbiotic biotrophy (Martin and Nehls, 2009; Kloppholz *et al*., 2011).

1.9.2 Highly expressed during infection

Effectors are highly expressed specifically during plant colonisation and infection (Rep, 2005). Although, some avirulence genes such as *Avr9* (from the tomato pathogen *C. fulvum*), *CgDN3* (from *Colletotrichum gloeosporioides* affecting Brazilian lucerne) and *MPG1* (from the rice blast fungus *Magnaporthe grisea*) are also induced *in vitro* during nitrogen starvation (Snoeijers *et al*., 1999, Stephenson *et al.*, 2000; Talbot *et al*., 1993).

1.9.3 Short, cysteine rich and secreted into the host apoplast

Effectors are secreted during infection either into the host apoplast (extracellular space, including xylem) or into the cytoplasm (intracellular space) (Kamoun, 2009).

Apoplastic effectors are generally small (<350 amino acid) cysteine rich proteins with a type II secretion signal at the N-terminal. Avirulence genes *Avr2*, *Avr4* and *Avr9* of *C. fulvum* and *EPI* genes of *Phytophthora infestans* can be mentioned as examples (reviewed by Kamoun, 2009). For example, *SnTox1* is a

necrotrophic effector (host-selective toxin) identified in the wheat pathogen *Stagonospora nodorum* that encodes a 117 amino acid protein containing 16 cysteine residues (Liu *et al*., 2012). The cysteine residues are important because disulphide bonds can stabilize the effector protein, once they are secreted into the apoplast.

1.9.4 Secreted into the host cytoplasm

Cytoplasmic effectors in fungi are difficult to identify, because they typically lack shared characteristics. Many are only predicted based on having a secretion signal. However others have been described that lack a signal peptide, such as in the cereal pathogen *Blumeria graminis* f. sp. *hordei* (Ridout *et al*., 2006). Only 60% of the Crinkler effectors (CRNs) of the potato pathogen *P. infestans* possess a predicted signal peptide (Haas *et al*., 2009) and they are not particularly short (>400 amino acid). The Pwl effectors, generally found in rice pathogens, are small, glycine-rich secreted proteins which confer avirulence on weeping lovegrass and finger millet (Kang *et al*., 1995).

1.9.5 Possess specific enzymatic or unknown functions

Sequences of effectors indicate enzyme functions such as host cell wall degradation (Avr4 effector of *C. fulvum*) or proteases (Avr9 effector of *C. fulvum*) (van Esse *et al*., 2007, van den Hooven *et al*., 2001). A few of them show similarity to other known effectors *e.g*. SnTox1 of *S. nodorum* shows a high level of similarity to the PtrToxA gene from *Pyrenophora tritici-repentis* as they both encode toxins which interact with the same locus in wheat (Friesen *et al*., 2008). However, a larger

proportion of known effectors have novel sequences, for example in *Colletotrichum higginsianum* (Kleemann *et al*., 2012).

1.9.6 Contain certain motifs or domains

Motifs have been identified from some of the effectors. Most oomycete effectors contain a RXLR-dEER (RXLR-effectors) motif which has a role in the delivery of the effector to the host (Whisson *et al*., 2007). CRN effectors of *P. infestans* are defined by the presence of LFLAK and DWL domains which have roles in effector translocation into the host (Birch *et al*., 2008). Analysis of the genome of *Hyaloperonospora arabidopsidis* revealed the presence of 134 potential effector genes containing HaRxLs (Baxter *et al*., 2010). Avr4 (*C. fulvum*) orthologues are present in many fungal species such as the cereal pathogen *Septoria tritici* (*Mycosphaerella graminicola*), the rice blast fungus *M. oryzae* and the wilt pathogen *Verticillum dahliae*, which can be identified based on the presence of LysM domain in all of them (Marshall *et al*., 2011, Klosterman *et al*., 2011, Mentlak *et al*., 2012). The Y/F/WxC motif discovered in *B. graminis* f. sp. *hordei* has been proposed to define a new class of effectors from haustoria-producing pathogenic fungi (Godfrey *et al*., 2010).

1.9.7 Encoded in a close proximity of other pathogenicity related genes

Pathogenicity related genes are often encoded on regions and even on entire chromosomes in fungal species. For example, the avirulence genes *AbrLm1* and *AvrLm6* of *Leptosphaeria maculans* are encoded in a GC-low, gene-poor,

heterochromatin-like region surrounded by a GC-rich region containing long-terminal-repeat (LTR) retrotransposons (van de Wouw *et al*., 2010). Moreover, host specific toxin genes of the tomato pathogen *Alternaria arborescens* are located on conditionally dispensable chromosomes (Hu *et al*., 2012).

1.9.8 Accumulated or secreted through haustoria, appressoria and infectious hyphae

Effectors are often accumulated and secreted through structures enabling entry into host such as haustoria, appressoria, and infectious hyphae. Highly expressed effector genes were identified from the appressoria of the *Brassica* pathogen *C. higginsianum* or rice blast fungus *M. oryzae* (Kleemann *et al*., 2008, O'Connell *et al*., 2012; Mentlak *et al*., 2012) and in the haustoria of *Melampsora lini* (Dodds *et al*., 2004).

1.10 Identification of fungal effectors

In 1942, Harold Henry Flor proposed his pioneering theory of gene-for-gene relationships based on his observations on the flax – *Melampsora lini* pathosystem (Flor, 1971). He proposed that for every dominant avirulence gene (*Avr*) in the pathogen there is a matching dominant gene (*R*) determining resistance in the host plant. At this time, the identification of avirulence genes was not possible due to the lack of methods such as cosmid cloning. The first avirulence gene, *avrA* of *Pseudomonas syringae* pv. *glycinea* was cloned by Staskawicz *et al*. in 1984. In 1991, the first fungal avirulence gene, *avr9* of *Cladosporium fulvum* was cloned by van Kan and his colleagues (van Kan *et al*., 1991). The first resistance gene, *Hm1* in maize to *Cochliobolus carbonum,* was identified by Johal and Briggs in 1992.

Map based cloning was the first approach used to identify avirulence genes, but since then many new technical discoveries have been made enabling faster identification of these genes. For instance, biochemical analysis of FOL infected tomato xylem sap led to the identification of the Six proteins (Rep, 2005). Similarly, small secreted proteins were identified by mass spectrometry-based proteomics from the haustoria of the barley pathogen *Blumeria graminis* f. sp. *hordei* (Bindschedler *et al*., 2009). Reverse genetics approaches, restriction enzyme mediated integration (REMI) and *Agrobacterium*-mediated transformation (ATMT) have also been successfully used to identify pathogenicity related genes (Rogers *et al*., 2004; Inoue *et al*., 2002). *Agrobacterium*-mediated insertional mutagenesis was used to identify 111 pathogenicity related genes of FOL (Michielse *et al*., 2009).

Recent advances in sequencing technologies and bioinformatics have accelerated the identification of (candidate) effector genes (Alfano, 2009). *In silico*

tools, such as SIGNALP, enable the identification of signal peptide sequences (Nielsen *et al*., 1997) and the latest version of this software is even capable of discriminating signal peptides from transmembrane regions (Petersen *et al*., 2011). This tool has revolutionised the prediction of secreted effectors from haustoria- or appressoria-specific expressed sequence tag (EST) libraries and has been used on fungi such as *Uromyces fabae, Phytophthora infestans, M. lini, Magnaporthe grisea* and *Colletotrichum higginsianum* (Kemen *et al*., 2005, Tian *et al*., 2004, Catanzariti *et al*., 2006, Dean *et al*., 2005, Kleemann *et al*., 2008).

Another *in vitro* method for the identification of secreted proteins is 'yeast-based signal sequence trap' which was successfully applied to *Phytophthora sojae* and *Uromyces fabae* (Lee *et al*., 2006; Link and Voegele, 2008). An exciting new method is deep RNA sequencing which enabled the identification of a single effector Ave1 of *Verticillium dahliae* corresponding to the tomato immune receptor Ve1 (de Jonge *et al*., 2012). The increasing number of genome sequences provides an ample opportunity to predict secreted protein and effector genes related to plant-associated species. This has been done successfully for *P. sojae*, *P. ramorum*, *Hyaloperonospora arabidopsidis, Albugo candida* and *Fusarium graminearum* (Win *et al*., 2007, Fabro *et al*., 2011; Saunders *et al*, 2012; Brown *et al*., 2012; Link *et al*., 2011).

Candidate effectors can also be identified by comparative genomics, looking at differences in GC composition or codon usage and searching for secretion signals, motifs or sequences similar to other effectors or sequences with no known homologues in other species (Alfano, 2009). Blast2GO software can be used for the rapid and automated annotation of predicted proteins which gives information on the similarity to known sequences and also about the presence of a secretion signal

(Conesa *et al*., 2005). The PHI-BASE database comprises a list of 1065 experimentally confirmed pathogenicity related genes of 97 pathogens (Winnenburg *et al*., 2008). This database provides an enormous resource for the identification of homologues of known pathogenicity related genes in genome sequences (van de Wouw and Howlett, 2011). The number of bioinformatics tools and sources is increasing which enables the identification of an increasing number of candidate effectors, aiding the future identification of effector targets in host plants (Saunders *et al*., 2012).

1.11 OBJECTIVES AND OUTLINE OF THE THESIS

The primary objective of this study was to characterise the diversity and the species and sub-species level identity of *Fusarium* species populations associated with diseased *Allium* and sweet pepper crops in the UK. No survey or study of *Fusarium* causing problems on these hosts has previously been done and there is a clear need to identify and characterise the species responsible to inform disease management strategies. Rapid and reliable detection and identification of potential plant pathogens is required to inform correct control measures (Lievens *et al*., 2008). Molecular methods offer invaluable solutions to facilitate the rapid detection and identification of pathogens. These methods also enable cultivation independently of the detection of pathogens, especially obligate pathogens and soil borne diseases where mixed populations are present (e.g. coexisting saprotrophic and pathogenic isolates of the same species).

The secondary objective of this project was therefore to identify molecular markers suitable for the development of a sensitive PCR diagnostic assay for detecting pathogenic *F. oxysporum* on onion sets and distinguish these from non-pathogenic isolates.

The first part of the thesis is the General Introduction in which overview of *Fusarium* genus with special focus of *F. oxysporum* are described. This chapter is followed by experiments focusing on molecular identification of *Fusarium* isolates associated with diseased *Allium* species in the UK and testing and development of primer pairs used for *Fusarium* identification. The third chapter contains information on pathogenic variation of *Fusarium* species associated with diseased *Allium* crops and on the characterisation of *F. oxysporum* isolates based on a set of housekeeping genes, genotyping markers and an effector gene. This chapter is followed by the

description and discussion of preliminary results on genome sequencing of *F. oxysporum* f. sp. *cepae*. The fifth chapter discussed results on molecular identification of *Fusarium* isolates associated with internal fruit rot of sweet pepper in the UK. The final chapter contains the Conclusions, the findings from all chapters are put into a wider context and the contribution of this project to the state of knowledge and future prospective are discussed.

CHAPTER 2

IDENTIFICATION OF *FUSARIUM* PATHOGENS CAUSING DISEASES ON *ALLIUM* CROPS IN THE UK

2.1 INTRODUCTION

Onion (*Allium cepa* L.) is an important horticultural crop grown worldwide and is sold as dry bulb. Dry onion was one of the 20 most important agricultural commodities, based on value, in the UK in the last 15 years (FAOSTAT, 2010). Over 350,000 tonnes of marketed onions are produced annually on about 8700 hectares in the UK. The main production regions are Lincolnshire, Essex, Suffolk, Cambridgeshire, Bedfordshire, Yorkshire and Kent (O`Connor, 2005).

The most common fungal pathogens which cause losses for UK onion producers are *Botrytis aclada, Botrytis squamosa, Peronospora destructor, Sclerotium cepivorum, Urocystis cepulae* and *Fusarium oxysporum* f. sp. *cepae* (Maude *et al*., 1984; Clarkson *et al*., 2000; Gilles *et al*., 2004; Coventry *et al*., 2002; Taylor *et al*., 2012). *F. oxysporum* f. sp. *cepae* W. C. Snyder & H. N. Hansen (FOC) was described as the causal agent of onion basal rot, a soil-borne vascular disease. Onion basal rot causes economic losses of around £10–11 million annually in the UK, and is therefore a high priority in the research strategy of the British Onion Producers Association (Alaister Findlay, BOPA, pers. comm.). Onion basal rot is mainly a problem for individual growers on particular fields and losses of up to 60% can occur (Andrew Richardson, Allium and Brassica Centre, pers. comm.).

F. oxysporum f. sp. *cepae* (FOC) can cause symptoms at every stage of the plant development and infects the roots and basal plate of sets and bulbs, causing a brown basal rot that becomes covered with a white-pink mycelium (Figure 2A). Leaf tips yellow, leaves wilt beginning with the older outer leaves and the plants die (Figure 2B). FOC can also cause seedling damping off, or delayed emergence of seedlings (Cramer, 2000).

Figure 2. Onion basal rot symptoms **A** Development of mycelium on basal plate and **B** wilting and yellowing.

FOC invades the roots or the stem plate of onion bulbs by direct penetration or through wounds, and then grows through intercellular spaces until invading the xylem vessels and xylem parenchyma. Xylem vessels blocked by tyloses are observed in both roots and, more commonly, in the stem plate. The pathogen can break through the stem area and grow in the fleshy leaf base tissues and between the leaves. Chlamydospores are formed in the root cortex and in and around the xylem vessels, while microconidia are produced by hyphae growing between the scale tissues in the bulb (Abawi and Lorbeer, 1971).

The pathogen can spread in stores through the wounds of dormant bulbs (Özer & Köycü, 2004) and within and between fields on equipment and infected plant material (Maude, 2006; Cramer, 2000). The pathogen can survive as chlamydospores (thick-walled survival structures) for up to 10 years in the soil or on soil debris as saprotrophic mycelium where onions are grown continuously (Entwistle, 1990). The disease is more serious when soil temperatures exceed 25°C and soil moisture is high.

FOC isolates show different levels of aggressiveness in onion seedlings, but separate races have not been identified (Cramer, 2000). FOC comprises seven known vegetative compatibility groups (VCGs 0420 to 0426) and several single-member VCGs (SMVs). However, it should be noted that the VCG analysis was carried out only on isolates collected in Japan, South Africa, Colorado (United States), and Turkey (Southwood *et al.*, 2012).

Recent studies from the Netherlands, Uruguay, Serbia, Turkey and Washington State, USA reported a complex of *F. oxysporum* and *F. proliferatum* (teleomorph: *Gibberella intermedia*) species causing onion basal rot. Additionally, in Turkey *F. solani* (teleomorph: *Nectria haematococca*), *F. acuminatum* (teleomorph: *G. acuminata*) and *F. redolens* were also found to be very aggressive in onion (Galvan *et al*., 2008; du Toit *et al*., 2003; Klokocar-Smit *et al*., 2008; Bayraktar *et al*., 2011). In the Netherlands *F. avenaceum* (teleomorph: *G. avenacea*) was identified as weakly pathogenic (de Visser *et al*., 2005). In an early study, *F. equiseti* (teleomorph: *G. intricans*), *F. verticillioides* (teleomorph: *G. moniliformis*) and *F. tricinctum* (teleomorph: *G. tricincta*) were also all found to be pathogenic to onion (Mannerucci *et al*., 1987).

Basal rot symptoms caused by FOC can occur on onion (*A. cepa*), shallot (*A. cepa* var. *ascalonicum*), Welsh onion (*A. fistulosum*), chive (*A. schoenoprasum*) leek (*A. ampeloprasum* var. *porrum*) and garlic (*A. sativum*) (Bayraktar *et al*., 2010; Coskuntuna & Özer, 2008; Özer & Köycü, 2004; Swift *et al*., 2002; Figure 3). From diseased garlic *F. proliferatum, F. verticillioides, F. culmorum* and from diseased Welsh onion *F. redolens, F. verticillioides, F. solani* have also been isolated (Dugan *et al*., 2007; Dissanayake *et al*., 2009ab; Seefelder *et al*., 2002; Stankovic *et al*., 2007; Shinmura, 2002; Palmero *et al*., 2012).

Leeks can suffer from foot and or basal rot depending on which *Fusarium* species has infected the plant. Foot rot is caused by *F. oxysporum* and *F. avenaceum* (Figure 3), while basal rot is caused by *F. culmorum* (Armengol *et al*., 2001; Koike *et al*., 2003; Gilardi *et al*., 2009; Oxspring *et al*., 2004).

Members of the wild *Allium* flora have not been reported as potential hosts of *F. oxysporum* in the UK. The wild species *A. roylei, A. galanthum,* and *A. pskemense* showed an intermediate level of resistance against *F. oxysporum* and *F. proliferatum* (Galvan *et al*., 2008). There is some information suggesting that FOC can colonize without causing symptoms economically important weed species such as oxalis (*Oxalis corniculata*) and fat-hen (*Chenopodium album*) (Abawi and Lorbeer, 1972).

As with many other soil-borne diseases, onion basal rot is difficult to control. Chemical control is rather limited in the UK, partly because thiram (dimethylcarbamothioylsulfanyl-N,N-dimethylcarbamodithioate) is the only recommended fungicide in the UK, and this can only be used for seed-treatment (Health and Safety Executive, HSE). The other reason is the increasing demand for organic products and more sustainable approaches to disease control. Biocontrol agents such as *Trichoderma* species, *Bacillus subtilis* and *Pseudomonas fluorescens*

were shown to be effective against onion basal rot (Coskuntuna and Özer, 2008; Cramer, 2000), but only *B. subtilis* (sold as Serenade ASO) is registered for onion treatment in the UK (Fungicide Resistance Action Group, FRAG). Work is in progress to optimise field-scale control of Fusarium basal rot and white rot of onion using *Trichoderma* amended substrates and pellets, and onion residues (Ralph Noble, East Malling Research, pers. comm.). Good management practices can help to prevent the occurance and spread of onion basal rot such as use of at least a 4-year-crop rotation, cleaning agricultural machinery between fields, temperature and humidity controlled-storage and soil solarisation (Klein *et al*., 2011). It is not clear why onion basal rot has become an increasing problem in the UK since 2006, but theories include the role of recent erratic weather, withdrawal of fungicides and emergence of new and/or more aggressive isolates or a combination of the above (A. Richardson, A&B Centre, pers. comm., A. Findlay, BOPA, pers. comm.).

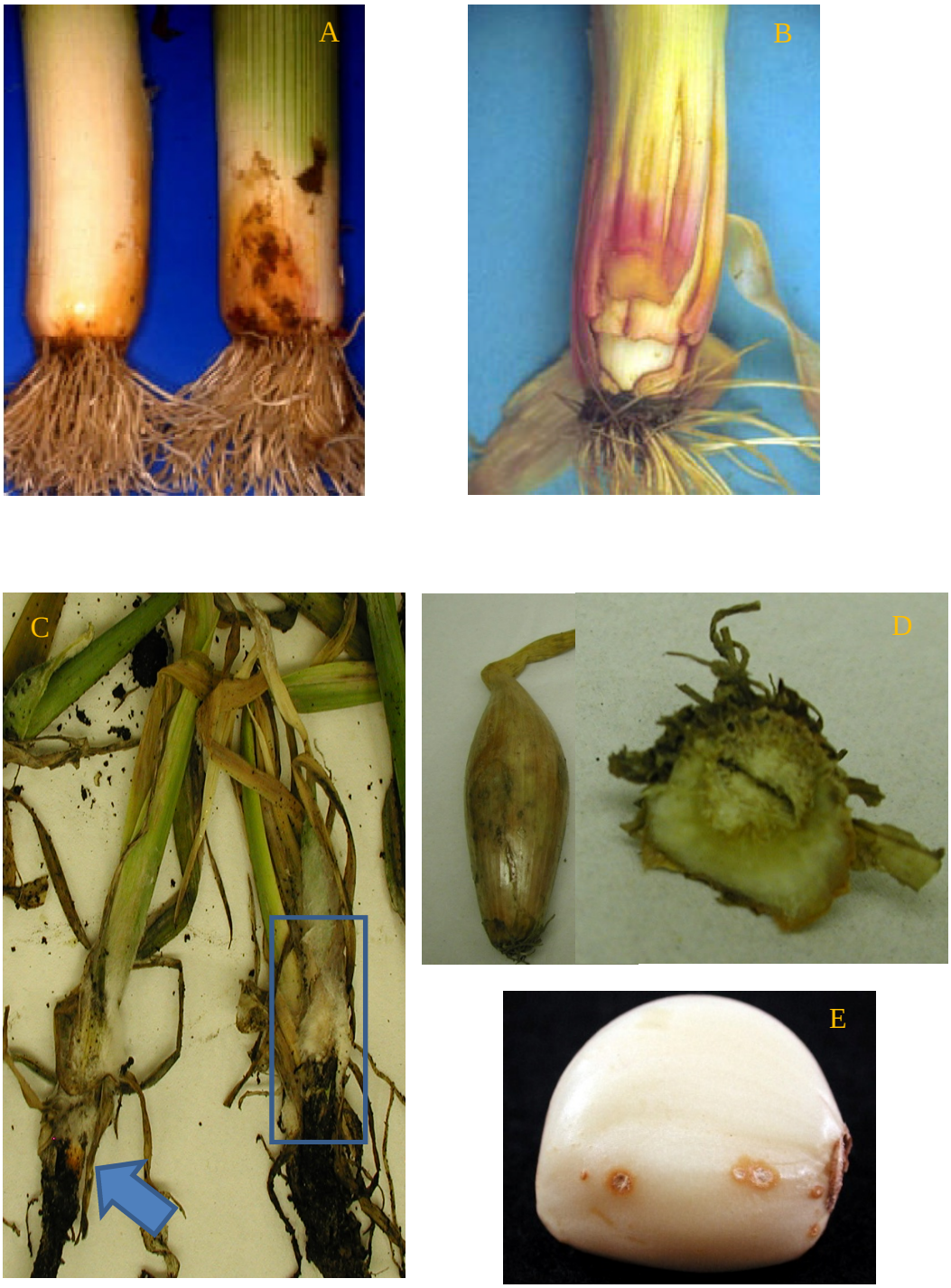


Figure 3. Fusarium rot symptoms of various *Allium* species. **A-B** Foot rot of leek (Photo credit: Hitch, C. J., SARDI) **A** *Fusarium avenaceum* causes a brown rot at the base of the shank. **B** *F. oxysporum* causes a pink discoloration on the shank. **C** Basal rot of leek, *F. culmorum* causes an orange discoloration on the shank and mycelial growth on leaves. **D** Basal rot of shallot **E** Basal rot of garlic (Photo credit: Putnam, M., Oregon State University).

2.2 AIMS AND OBJECTIVES

The overall aim of this chapter was to identify the species and sub-species of *Fusarium* populations on *Allium* crops in the UK and characterise their diversity.

Specific objectives were:

- To obtain *Fusarium* isolates from soil and *Allium* crops with basal rot symptoms
- To identify and characterise *Fusarium* species using genus- and species-specific primers and amplifying a common fungal housekeeping gene (*TEF*).

2.3 MATERIALS AND METHODS

2.3.1 Testing and development of primer pairs used for *Fusarium* identification

2.3.1.1 DNA extraction

DNA was extracted from 22 type cultures including *F. avenaceum, F. begoniae, F. cerealis, F. coeruleum, F. culmorum, F. equiseti, F. foetens, F. fujikuroi, F. graminearum, F. lateritium, F. oxysporum* f. sp. *tulipae, F. poae, F. proliferatum, F. redolens, F. sambucinum, F. solani, F. succisae, F. torulosum, F. tricinctum, F. verticillioides, Colletotrichum gloeosporioides* and *Microdochium nivale* (Appendix I) using a GenElute Plant Genomic DNA Kit (Sigma-Aldrich) following the producers protocol.

2.3.1.2 Polymerase chain reactions (PCRs) amplifying part of the *TEF* gene and ITS region for the confirmation of species level identity of *Fusarium* type cultures

Species level identity of the 20 type *Fusarium* cultures was confirmed by amplification and sequencing of the partial translation elongation factor 1α (*TEF*) gene, commonly used for fungal identification studies, using EF1 and EF2 primer pair and the cycling conditions published by O'Donnell *et al*. (1998a). Two closely related species, *C. gloeosporioides* and *M. nivale*, were identified based on ITS region using ITS1 and ITS4 primers and thermocycler conditions published by White *et al*. (1990). PCR reactions were carried out in a total volume of 20 µl which consisted of: 10 µl of RedTaq (Sigma-Aldrich*),* 7 µl of sterile water, 1 µl each of 20 µM forward/reverse primers and 1 µl DNA template (30 ng/µl).

PCR products were electrophoresed on 1.0 w/v % agarose gels in TAE buffer with 1 µl of GelRed (Biotium) per ml following standard procedures (Sambrook *et al*., 1989).

Sequencing reactions were carried out in a total volume of 10 µl which consisted of: 2 µl of Big Dye terminator 3.1 (Applied Biosystems, ABI), 2 µl of 5x sequencing buffer (ABI), 4 µl of sterile water, 1 µl of 4 µM forward/reverse primer and 1 µl PCR amplicon (200 bp PCR product: 30 ng/µl; 200-1000 bp product: 10 ng/µl) using an ABI Prism 3100 Genetic Analyser. Thermal cycling conditions were as follows: initial denaturation for 1 minute at 96°C, followed by 25 cycles of 10 seconds denaturation at 96°C, annealing for 5 seconds at the 50°C and extension for 3 minutes at 60°C. The program ended with a 4 minutes final extension at 60°C.

Basic editing of sequence files and preparation of consensus sequences was done using DNASTAR Lasergene 8.0. Sequences were compared with those deposited of National Center for Biotechnology Information (NCBI) by using the Basic Local Alignment Search Tool (BLAST; Altschul *et al*., 1990).

2.3.1.3 Testing of published primers pairs for *Fusarium* genus and species identification

For the rapid identification of fungal isolates recovered from *Allium* species and soil associated with basal rot, *Fusarium* genus and species-specific primer pairs were used. Compared to sequence based identification the use of specific PCR reactions is cheaper and faster. *F. oxysporum* and *F. proliferatum* have been recovered most frequently in association with onion basal rot in several countries (Galvan *et al*., 2008, du Toit *et al*., 2003, Klokocar-Smit *et al*., 2008, Bayraktar *et al*., 2011) and therefore specific primer pairs designed for the detection of these species were also tested. In total, two *Fusarium* genus-specific (Bluhm *et al*., 2004;

Abd-Elsalam *et al*., 2003), a *F. oxysporum*-specific (Mule *et al*., 2004) and a *F. proliferatum*-specific (Mule *et al*., 2004) primer pairs were initially tested on *F. redolens, F. culmorum, F. proliferatum, F. solani, F. oxysporum* f. sp. *tulipae*, *F. avenaceum, F. equiseti,* and *M. nivale* (see 2.3.1.1; Table 1). The published *Fusarium* genus-specific primers targeted the ITS region, while species specific primer pairs were designed based on calmodulin coding sequences (Bluhm *et al*., 2004; Abd-Elsalam *et al*., 2003; Mule *et al*., 2004).

PCR reactions were carried out in a total volume of 20 µl which consisted of: 10 µl of RedTaq (Sigma-Aldrich), 7 µl of sterile water, 1 µl each of 20 µM forward/reverse primers and 1 µl DNA template (30 ng/µl). Thermocycling conditions were as published (Bluhm *et al*., 2004; Abd-Elsalam *et al*., 2003; Mule *et al*., 2004).

Table 1. Sequences, target gene, specificity and origin of primers used for the identification of fungal isolates.

Primer name	Primer sequence (5`→3`)	Target	Specificity	Reference
ITS-Fu-f	CAACTCCCAAACCCCTGTGA	ITS region	*Fusarium* genus	Abd-Elsalam *et al*., 2003
ITS-Fu-r	GCGACGATTACCAGTAACGA			
FuITS Forward	AACTCCCAAACCCCTGTGAACATA	ITS region	*Fusarium* genus	Bluhm *et al*., 2004
FuITS Reverse	TTTAACGGCGTGGCCGC			
CLOX1	CAGCAAAGCATCAGACCACTATAACTC	calmodulin gene	*Fusarium oxysporum*	Mule *et al*., 2004
CLOX2	CTTGTCAGTAACTGGACGTTGGTACT			
CLPRO1	TGCATCAGACCACTCAAATCCT	calmodulin gene	*Fusarium proliferatum*	
CLPRO2	GCGAGACCGCCACTAGAT			

2.3.1.4 Development of novel a primer pair for the identification of isolates belonging to the *Fusarium* genus

A *Fusarium* genus-specific primer pair (exTEFfor and FUexTEFrev) targeting the *TEF* gene was designed to speed up the process of identification as *TEF* is the most widely used marker for *Fusarium* species identification. The *Fusarium* genus-specific primer pair was designed manually based on the *TEF* gene sequences of twelve fungal genomes (*F. graminearum. F. oxysporum, F. verticillioides, F. solani, Trichoderma reesei, T. atroviridae, T. virens, Epichloë festucae, Glomerella graminicola, Verticillium dahliae, Aspergillus nidulans,* and *A. terreus*) (BROAD, JGI). Self- and cross-hybridisation ability was tested by Oligo Analysis Tool (Eurofins). Primer specificity was tested *in silico* using NCBI BLAST tool (Altschul *et al*., 1990). All primers were synthesised by Invitrogen (Paisley, UK).

Specificity of exTEFfor and FUexTEFrev primers was tested on the 22 type cultures as listed in Section 2.3.1.1 using the same PCR reaction as in Section 2.3.1.2. Thermocycling conditions were as follows: initial denaturation for 5 minute at 94°C, followed by 35 cycles of 45 seconds denaturation at 94°C, annealing for 30 seconds at the 64°C and extension for 2 minutes at 72°C. The program ended with a 10 minutes final extension at 72°C. Species level identity of all *Fusarium* isolates was confirmed by sequencing the *TEF* gene using EF1 and EF2 primers (O'Donnell *et al*., 1998a) as in Section 2.3.1.2.

2.3.2 Sampling and isolation of *Fusarium* species associated with *Allium* species in the UK

Onion bulbs showing basal rot symptoms were collected from stores or directly from fields. Field samples were taken by walking diagonally across each field and taking bulb samples with symptoms of basal rot from at least ten plants per a field (Table 2). Approximately 1 cm^3 sections of diseased and healthy basal tissue of *Allium* samples (Table 2) were surface sterilised (twice in 70% ethanol for 5 minutes each followed by two rinses in sterile distilled water) and transferred onto water agar (20 g agar in 1L of distilled water) amended with antibiotics (1 g streptomycin sulphate and 0.12 g neomycin per L media to inhibit bacterial growth) and incubated at 25°C for 3 days. Isolates were sub-cultured onto potato dextrose agar (PDA, Merck) via hyphal tip transfer to obtain pure cultures. After 7-10 days incubation, isolates with different colony morphologies characteristic of *Fusarium* were selected for further studies.

Fusarium isolates (168) were recovered from onion bulbs showing basal rot symptoms from 17 samples collected in the UK (Table 2). Imported onion bulbs and sets were also used for isolations (35 isolates) to investigate the possibility of new *Fusarium* isolates being introduced into the UK from other countries (Table 2). These were imported originating from the Netherlands, Belgium and France, while imported onion bulbs were from Spain, the Netherlands, Chile and New Zealand. Garlic, shallot and leek plants were collected from production and processing sites in the UK in order to define which *Fusarium* species were present on other *Allium* crops (Table 2). Nineteen isolates were obtained from these.

Additionally, *Fusarium* isolates (77) were also obtained following isolations from soil samples from three field sites (Table 2, Figure 4). Soil samples (Table 2)

were air dried and sieved before plating on peptone PCNB agar (PPA) and Komada`s media (Nash & Snyder, 1962; Komada, 1975). To obtain individual *Fusarium* colonies, 1 $x10^{-2}$ and 1 $x10^{-3}$ dilutions were used (starting with 1 g of soil in 10 ml sterile distilled water). After 10 days isolates were sub-cultured onto PDA.

A field in Warwickshire (Table 2, sample reference 17) was chosen for sampling as the onions recovered showed basal rot symptoms (Figure 4A). Five soil samples (1 – 5) were collected from this field. A second field (Table 2, sample reference 6) in Suffolk showed severe basal problems at the half of the field (Figure 4B). Five soil samples were collected from both symptomless (1 - 5) and diseased (6 - 10) bulbs. A third field in Essex (Table 2, sample reference 8) was sampled three years after onions were grown and also a year before the next onion crop was due to be grown (Figure 4C). The cropping history of the field was well documented; onion was grown in 4-year-rotation with winter wheat and sugar beet. Five samples (11 – 15) were collected. The same onion field in Essex (Table 2, sample reference 8) was sampled intensively shortly before harvesting in September 2010. To study the spatial variation of *Fusarium* species in these fields, a total of 70 bulbs were taken from positions approximately 10 m apart by walking diagonally and transversally across the field. The area sampled had a long history of onion basal rot and the first incidence of the disease in the 1980's was reported in the same region and by the same grower. A four-year crop rotation was used to grow onions on this field in alternation with winter wheat and sugar beet.

All *Fusarium* isolates were stored on Spezieller Nährstoffarmer Agar (SNA, Nirenberg, 1976) at 4°C or as spore suspension in 15% glycerol-water solution at -80°C.

Table 2. Origin, date of collection, source, host, sample material and number of isolates obtained of samples collected for isolation of *Fusarium* species.

Reference number	Location of onion field	Date of collection	Source	Host	Sample material	No. of isolates
1	Bedfordshire	Jul-10	stores	onion	bulb	4
2	Bedfordshire	Oct-09	stores	onion	bulb	6
3	Bedfordshire	Oct-09	stores	onion	bulb	4
4	Bedfordshire	Oct-09	stores	onion	bulb	12
5	Essex	Mar-09	stores	onion	bulb	8
6	Essex	Oct-09	stores	onion	bulb	10
7	Essex	Aug-10	field	onion	bulb	49
8	Essex	Sep-10	field	onion	bulb	31
9	Suffolk	Oct-09	stores/field	onion	bulb	5
10	Suffolk	Jun-09	stores	onion	bulb [c]	7
11	Suffolk	Dec-09	stores	onion	bulb	5
12	Suffolk	Jul-09	field	onion	bulb	7
13	Suffolk	Nov-10	stores	onion	bulb	7
14	Lincolnshire	Mar-09	field	onion	bulb	4
15	Lincolnshire	Aug-10	stores	onion	bulb	1
16	Nottinghamshire	Oct-09	stores	onion	bulb	2
17	Warwickshire	Dec-08	stores	onion	bulb	6
17	Warwickshire [a]	Mar-09	field	onion	soil	36
6	Suffolk	Oct-09	field	onion	soil	24
8	Essex [b]	Oct-09	field	onion	soil	17
18	The Netherlands	2009	stores	onion	set	5
19	France	2009	stores	onion	set [c]	1
20	Belgium	2009	stores	onion	set [c]	1
21	New Zealand	2009	stores	onion	bulb	1
22	The Netherlands	2010	stores	onion	bulb	4
23	Spain	2010	stores	onion	bulb	16
24	Chile	2011	stores	onion	bulb	7
25	Cambridgeshire	Jun-10	glasshouse	leek	stalk	8
26	Bedfordshire	Nov-10	store	garlic	cloves	8
27	Bedfordshire	Nov-10	store	shallot	bulb	3
Total						299

[a]:Year after onion crop grown, [b] Year before onion was grown, wheat field at the time of sampling [c]: plant material did not show symptoms of onion basal rot

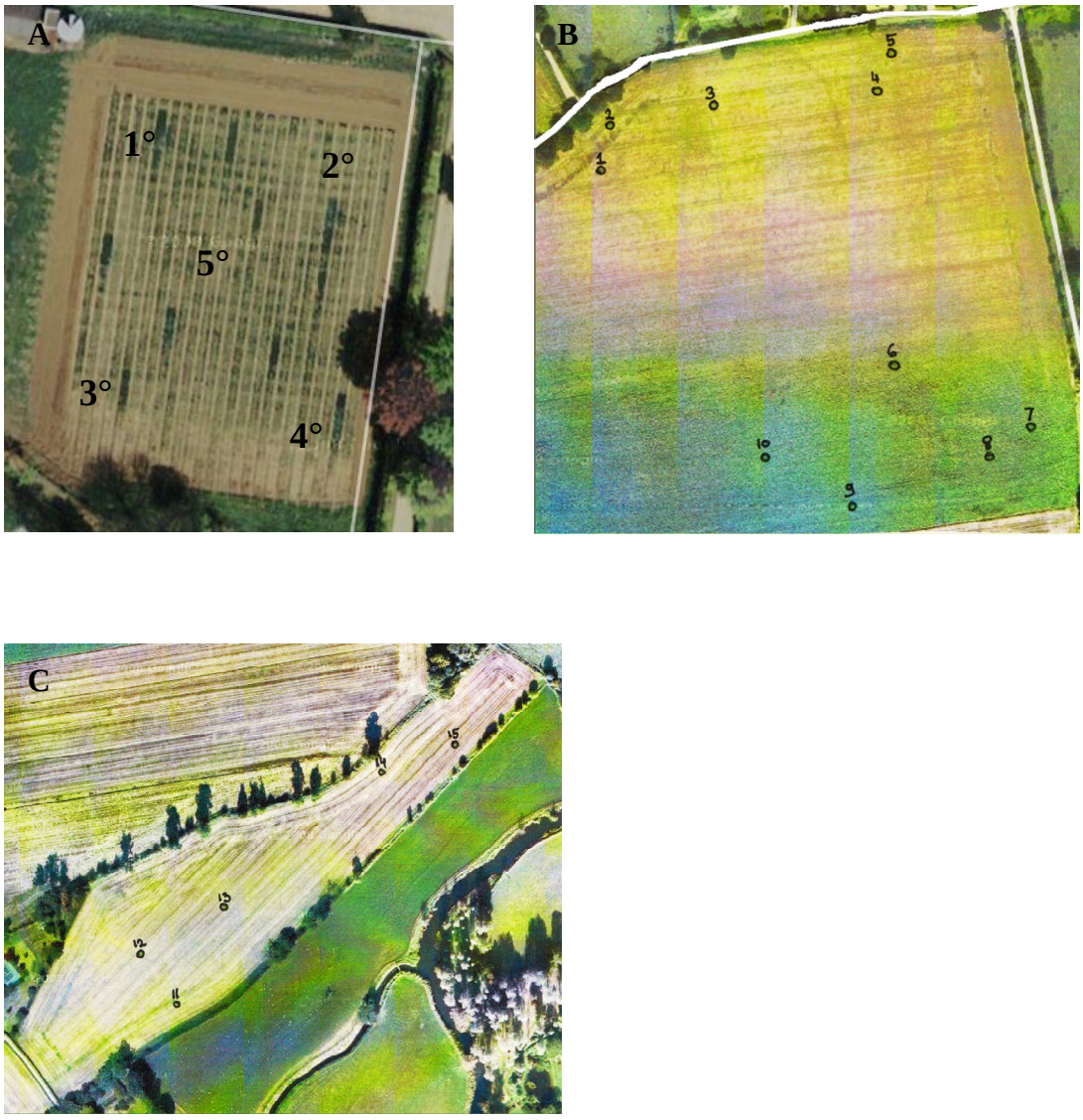


Figure 4. Soil sampling points (marked with circles) of three onion fields with onion basal rot incidence. **A** Five soil samples (1 - 5) were collected from field in Warwickshire with severe basal rot incident (reference number 17); **B** Field in Suffolk (reference number 6), onions showing onion basal rot symptoms were found only from half of the field (shaded in green). Five samples were collected from symptomless half (1 – 5) on the field and five samples (6 – 10) were collected from diseased half of the field; and **C** Five samples (11 - 15) were a field in Essex (reference number 8) with onion basal rot history.

2.3.3 Molecular identification of *Fusarium* isolates associated with *Allium* species

2.3.3.1 DNA extraction method used for the preliminary identification of *Fusarium* isolates

For preliminary identification of isolates, DNA was extracted by a method using 10% Chelex 100 solution adapted from Richlen and Barber (2005) and Talhinhas *et al*. (2009). Chelex 100 is an ion chelator which protects DNA molecules, removes magnesium from the sample and inactivates nucleases. The protocol involved the preparation of sterile 1.5 ml Eppendorf tubes containing 10% Chelex 100 (0.1 g Chelex 100 and 900 µl dH_2O). A loopful of mycelium was collected from 10-day-old cultures grown on PDA plate with a sterile cocktail stick. Mycelium was transferred into 1.5 ml Eppendorf tubes containing 10% Chelex 100 and vortexed for 30 seconds followed by 30 seconds centrifugation at 14000 rpm. Tubes were incubated at 95˚C for 20 minutes, then vortexed for 10 seconds followed by 10 seconds centrifugation for at 14000 rpm. DNA-containing supernatant was transferred into a sterile Eppendorf tube and stored at -20°C.

2.3.3.2 Molecular identification of *Fusarium* isolates associated with *Allium* species

Preliminary identification of isolates obtained from *Allium* species and soil (Table 2) employed *Fusarium* genus- and species-specific primer pairs (exTEFfor, FUexTEFrev, CLOX1, CLOX2, CLPRO1 and CLPRO2 primers) using published conditions as in Sections 2.3.1.3 and 2.3.1.4. Species-level identity of 234 isolates (Appendix II and Appendix III) obtained from *Allium* species and soil was confirmed by sequencing part of the *TEF* gene as in Section 2.3.1.4.

2.3.3.3 Cladistic analysis of *Fusarium* isolates associated with *Allium* species

TEF sequences from *Fusarium* isolates were aligned by Clustal W (Thompson *et al*., 1994) and cladistic analysis carried out based on neighbour-joining (Saitou & Nei, 1987), maximum parsimony and UPGMA (Sneath and Sokal, 1973) using MEGA 4.0 (Tamura *et al*., 2007). Bootstrap consensus trees were inferred from 1000 replicates and are taken to represent the evolutionary history of the taxa (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Distances were computed using the Kimura 2-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Published *TEF* sequences of *F. oxysporum* f. sp. *cepae*, *F. proliferatum*, *F. avenaceum* and *F. culmorum* were used as references (Table 3; Mbofung *et al*., 2007; Galvan *et al*., 2008; O'Donnell *et al*., 2009).

Table 3. Genbank accession numbers of translation elongation factor 1α (*TEF*) sequences, origin, year of collection, reference and distribution of *Fusarium* species pathogenic towards *Allium* spp. used for taxonomic reference.

Accession number	Origin	Year	Reference	Species
DQ837681	Germany	1935	Mbofung *et al*., 2007	*F. oxysporum* f. sp. *cepae*
EU220393	USA, California	n.i.	Galvan *et al*., 2008	
EU220394	Australia	2004	Galvan *et al*., 2008	
EU220395	USA, New Mexico	2004	Galvan *et al*., 2008	
EU220396*	Spain	2004	Galvan *et al*., 2008	
EU220397	Unknown	1925	Galvan *et al*., 2008	
EU220398	The Netherlands	2004	Galvan *et al*., 2008	
EU220399	Uruguay	2004	Galvan *et al*., 2008	
EU220402	Turkey	n.i.	Galvan *et al*., 2008	
EU220404	The Netherlands	2004	Galvan *et al*., 2008	
FJ985399	New Zealand	1960	O`Donnell *et al*., 2009	
EU220405	The Netherlands	2004	Galvan *et al*., 2008	*F. proliferatum*
EU220406	Argentina	2004	Galvan *et al*., 2008	
EU220407	The Netherlands	2004	Galvan *et al*., 2008	
EU220408	Uruguay	2004	Galvan *et al*., 2008	
EU220410	The Netherlands	2004	Galvan *et al*., 2008	*F. culmorum*
EU220414	The Netherlands	2004	Galvan *et al*., 2008	*F. avenaceum*

2.4 RESULTS

2.4.1 Testing and development of primer pairs used for *Fusarium* identification

The specificity of published *Fusarium* genus- and species-specific primer pairs was tested on eight *Fusarium* cultures representing commonly found species in the UK and *M. nivale,* a species closely related to *Fusarium* genus, was used as a negative control.

The *Fusarium*-specific primer pair FuITS (Bluhm *et al*., 2004) were more reliable when used with *F. redolens* and *F. avenaceum* compared to the ITSFu pair which resulted in very faint bands (Abd-Elsalam *et al*., 2003; Table 4).

F. oxysporum specific PCR primers (Mule *et al*., 2004) were potentially suitable for distinguishing *F. oxysporum* from other *Fusarium* species, but needed to be used carefully as some of the other species also yielded PCR products with these primers but with different banding patterns (Table 4). The *F. proliferatum* specific PCR primers (Mule *et al*., 2004) were suitable for identifying isolates of this species as it gave a single bright band with *F. proliferatum* but not with any other species (Table 4).

The newly developed *Fusarium* genus-specific primer pair, exTEFfor ACCCGGTTCAAGCATCCGATCTGCGA and FUexTEFrev AGCTTGCCR GACTTGATCTCACGCTC was designed based on the *TEF* gene sequences of eight fungal genomes representing closely related genera (*Trichoderma, Epichloë, Glomerella, Verticillium* and *Aspergillus*). FUexTEFrev primer was found to be specific exclusively to *Fusarium* genus *in silico* when its specificity tested on the whole genome sequences of eight closely related fungal species. This novel primer pair was tested on 20 *Fusarium* species representing major plant pathogens and on

closely related *C. gloeosporioides* and *M. nivale* species. ExTEFfor and FUexTEFrev primers were able to amplify ~1269 bp product from all *Fusarium* species but not the two related species, *C. gloeosporioides* and *M. nivale* species (Figure 5).

In summary, two *Fusarium* genus-specific (both targeting the ITS region), a *F. oxysporum*-specific and a *F. proliferatum*-specific primer pair (both targeting calmodulin gene) were tested on a small set of isolates. Only one of the genus-specific (designed by Bluhm *et al*., 2004) and the two species-specific (published by Mule *et al*., 2004) primer pairs gave reliable results. A novel primer pair (exTEFfor and FuexTEFrev) targeting *TEF* gene was developed and tested successfully to identify/detect *Fusarium* species associated with onion basal rot.

Table 4. Primer efficiency in *Fusarium* genus-, *F. oxysporum*- and *F. proliferatum*-specific PCR screening.

Species	Isolate	FU-ITS[1]	ITS-FU[2]	CLOX[3]	CLPRO[3]
F. redolens	A18-2-5	+++	–	–	–
F. culmorum	C14-2-5	+++	+++	M	–
F. proliferatum	IMI 312182	+++	+	–	+++
F. solani	R161AF4	+++	+++	–	–
F. oxysporum	Fus 1	+++	+++	+++	–
F. avenaceum	2308-1b	+++	+++	–	–
F. equiseti	C-3-1-2	+++	+	M	–
M. nivale	08251	–	–	–	–

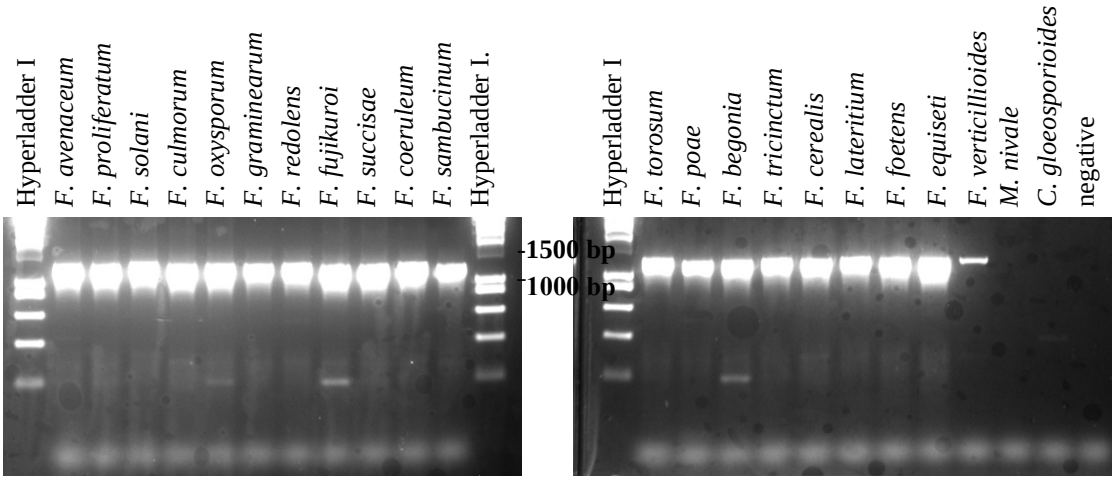


Figure 5. Banding pattern for the *Fusarium* genus-specific exTEFfor and FuexTEFrev primers tested on 20 *Fusarium* species, *Microdochium nivale* and *Colletotrichum gloeosporioides*. *M. nivale* and *C. gloeosporioides* were used as controls to test specificity of this reaction. ~1269 bp long product was amplified for all *Fusarium* species. Hyperladder I (Bioline) 10,000 bp molecular size marker was included in the first lane of each gel.

2.4.2 Characterisation of *Fusarium species* associated with *Allium* species in the UK

2.4.2.1 Distribution of isolates associated with basal rot of *Allium* species

The 10% Chelex 100 DNA extraction method (see Section 2.3.3.1) was suitable for PCR-based analysis, including amplification of single-copy targets (e.g. *TEF*). It is a quicker way of DNA extraction compared to the commercially available kits (from Qiagen and Sigma), but gave lower yield and quality of DNA (data not shown).

The *Fusarium* genus-specific primer pair (exTEFfor and FUexTEFrev) was used for primary classification of isolates which led to the identification of 299 *Fusarium* isolates (Table 5). The use of species-specific primer pairs (CLOX1/CLOX2 and CLPRO1/CLPRO2) revealed that the majority of isolates (205) obtained from the plant and soil samples collected were *F. oxysporum,* while only 15 isolates were identified as *F. proliferatum* (Table 5). The application of *F. oxysporum*- and *F. proliferatum*-specific primer pairs enabled the species level identification of 220 isolates, which left 78 isolates to be identified.

Table 5. Identification and distribution of *Fusarium* isolates from plant and soil samples associated with basal rot of *Allium* species.

Origin of samples	Year	Host	No. of isolates	*Fo* a	*Fp* a	*Fs* b	*Fr* b	*Fc* b	*Fe* b	*Fav* b	*Fac* b	*F*.sp. c
Field 1, Bedfordshire	2010	onion	4	4	0	0	0	0	0	0	0	0
Field 2, Bedfordshire	2009	onion	6	6	0	0	0	0	0	0	0	0
Field 3, Bedfordshire	2009	onion	4	4	0	0	0	0	0	0	0	0
Field 4, Bedfordshire	2009	onion	11	7	2	1	0	0	0	0	0	1
Field 5, Essex	2009	onion	8	5	0	1	2	0	0	0	0	0
Field 6, Essex	2009	onion	10	9	0	1	0	0	0	0	0	0
Field 7, Essex	2010	onion	49	47	0	0	2	0	0	0	0	0
Field 8, Essex	2010	onion	31	28	0	0	3	0	0	0	0	0
Field 9, Suffolk	2009	onion	5	3	0	1	0	0	0	0	0	1
Field 10, Suffolk	2009	onion	7	3	0	1	0	0	0	1	0	2
Field 11, Suffolk	2009	onion	5	3	0	0	1	0	0	0	0	1
Field 12, Suffolk	2009	onion	7	1	0	3	3	0	0	0	0	0
Field 13, Suffolk	2010	onion	7	7	0	0	0	0	0	0	0	0
Field 14, Lincolnshire	2009	onion	4	1	2	1	0	0	0	0	0	0
Field 15, Lincolnshire	2010	onion	1	1	0	0	0	0	0	0	0	0
Field 16, Nottinghamshire	2009	onion	2	1	0	0	0	0	0	0	0	1
Field 17, Warwickshire	2008	onion	6	3	3	0	0	0	0	0	0	0
Field 17, Warwickshire	2009	onion soil	36	19	0	0	0	0	0	0	0	17
Field 11, Suffolk	2009	onion soil	24	17	0	3	1	0	0	0	0	3
Field 8, Essex	2009	onion soil	17	2	0	0	3	1	3	0	0	8
The Netherlands	2009	onion set	5	5	0	0	0	0	0	0	0	0
France	2009	onion set	1	0	0	0	0	0	0	1	0	0
Belgium	2009	onion set	1	1	0	0	0	0	0	0	0	0
New Zealand	2009	onion	1	1	0	0	0	0	0	0	0	0
The Netherlands	2010	onion	4	4	0	0	0	0	0	0	0	0
Spain	2010	onion	16	8	6	0	0	0	0	1	1	0
Chile	2011	onion	7	6	1	0	0	0	0	0	0	0
Cambridgeshire	2010	leek	8	0	0	0	0	6	0	2	0	0
Bedfordshire	2010	garlic	8	6	1	1	0	0	0	0	0	0
Bedfordshire	2010	shallot	3	3	0	0	0	0	0	0	0	0
Total no. of isolates from plant material			222	167	15	10	10	6	0	5	1	6
Total no. of isolates from soil			77	38	0	3	4	1	3	0	0	28
Total			299	205	15	13	15	7	3	5	1	34

Fo: F. oxysporum, Fp: F. proliferatum, Fs: F. solani, Fr: F. redolens, Fc: F. culmorum, Fe: F. equiseti, Fav: F. avenaceum, Fac: F. acuminatum and *F*. sp.: *Fusarium* species

a: All isolates were identified by *Fusarium* genus- *F. oxysporum*- and *F. proliferatum*-specific primer pairs, additionally some isolates were also identified by *TEF* sequence similarity.

b: All isolates were identified by *Fusarium* genus specific primer pair and additionally some isolates were also identified at species level by *TEF* sequence similarity.

c: Isolates not been identified at species level

Of these 78 isolates, 44 were chosen based on origin and colony morphology for species-level identification by sequencing the *TEF* PCR products. Sequence comparison revealed the presence of *F. solani* (13 isolates), *F. redolens* (15 isolates), *F. culmorum* (7 isolates), *F. equiseti* (3 isolates), *F. avenaceum* (5 isolates) and *F. acuminatum* (1 isolate). Additionally, the identity of all *F. proliferatum* and 175 *F. oxysporum* isolates associated with *Allium* species was also confirmed by *TEF* sequencing (Appendix II). This approach enabled intra- and inter-specific genetic variation among *Fusarium* species associated with *Allium* species to be investigated. The remaining 34 *Fusarium* isolates associated with basal rot of *Allium* species isolates were not identified at species level.

F. oxysporum distribution

F. oxysporum was present in all mature onion, garlic, shallot samples, Dutch and Belgian sets and soil samples, but was not found in the leek samples or the French sets (Table 5). It was abundant in mature onion bulbs at every geographical location sampled including 16 fields across six English counties: Warwickshire, Bedfordshire, Essex, Suffolk, Lincolnshire and Nottinghamshire (Figure 6). *F. oxysporum* was also present in the Dutch and Belgian sets and the onion bulbs imported to the UK from four countries (Spain, Chile, the Netherlands and New Zealand).

F. proliferatum and *F. solani* distribution

F. proliferatum (15 isolates) was only isolated from onion plants in three different fields in the UK (Warwickshire, Bedfordshire and Lincolnshire) and from onion and garlic imported from Spain and Chile. *F. solani* (13 isolates) was found in

five onion samples (Bedfordshire, Essex, Suffolk and Lincolnshire) and from garlic and from soil collected in Suffolk (Figure 6).

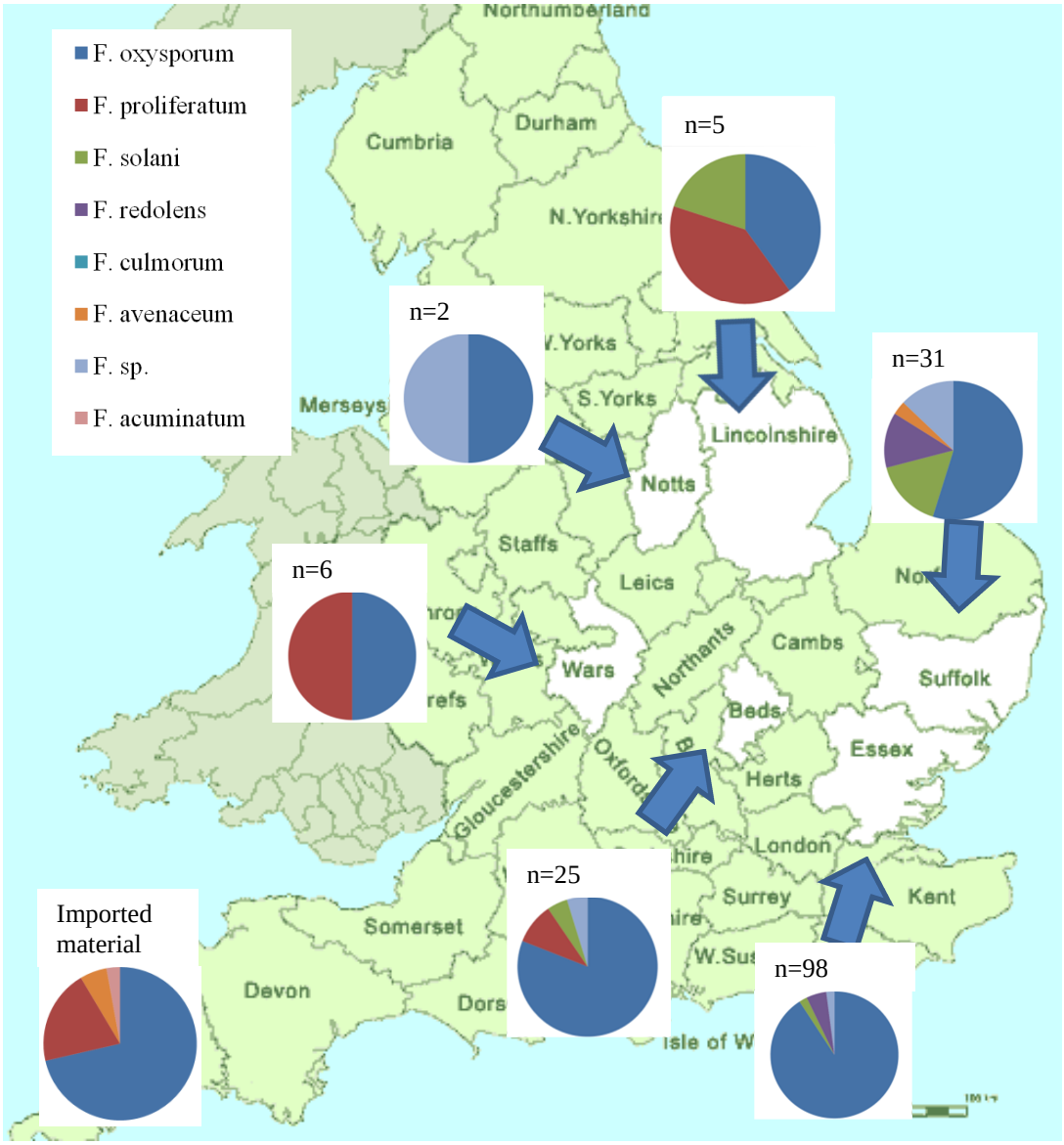


Figure 6. Origin and frequency of *Fusarium* species isolated from onion bulbs and sets in this study. Size of circles is not representative of the number of isolates obtained. Number of isolates (n) is shown next to corresponding pie chart.

F. redolens, F. avenaceum, F. acuminatum, F. culmorum and F. equiseti distribution

F. redolens (15 isolates) was recovered from soil and onions grown in Essex and Suffolk (Table 5; Figure 6). *F. avenaceum* (5 isolates) was recovered from

onions grown in Suffolk, Spanish onions, British leeks and French onion sets. One isolate of *F. acuminatum* was obtained from Spanish onions and one *F. culmorum* isolate was found in soil collected from a wheat field in Essex. Three isolates of *F. equiseti* were found in a soil sample collected in Essex.

Distribution of *Fusarium* species based on host and sample material

Six isolates obtained from leek were identified as *F. culmorum* and two as *F. avenaceum* (Table 5). Only *F. oxysporum* was found in shallot bulbs, while *F. oxysporum, F. solani* and *F. proliferatum* were found on garlic. *F. oxysporum* was identified in imported Dutch and Belgian sets and all imported onion bulbs (Table 5). Only one isolate of *F. avenaceum* was recovered from the French sets. The majority of the Chilean isolates (6) belong to *F. oxysporum* and only one isolate was identified as *F. proliferatum*. The Spanish onion batch was contaminated with *F. oxysporum* (8 isolates), *F. proliferatum* (6 isolates), *F. avenaceum* (1 isolate) and *F. acuminatum* (1 isolate).

***Fusarium* species from soil samples**

F. oxysporum was detected in every soil sample collected from fields where onions were grown either at the time of sampling or prior to sampling (Table 5). However, *F. proliferatum* was not present in any of the soil samples based on the results of species-specific PCR. Sequencing of the *TEF* gene also revealed the presence of *F. solani, F. redolens, F. culmorum, F. equiseti* and *F. avenaceum* in the soil of onion growing areas. A high proportion of isolates (53%) was identified as *F. oxysporum* from soil samples collected from a field in Warwickshire where onions were grown during the previous year (Table 6). A field in Suffolk (sample reference

6) was sampled a month after onion harvest in October 2009 where disease severely affected one half of the field (Table 6). Isolations from five soil samples (samples 1- to 5) collected from the symptomless part of the field led to the identification of two only *F. oxysporum* isolates from a total of six *Fusarium* isolates (33%). In contrast, 18 *Fusarium* isolates were obtained from the five soil samples collected from the diseased part (samples 6- to 10) of the field and 17 of them of them (88%) were identified as *F. oxysporum* (Table 6). *F. oxysporum* was less abundant (two out of 17 isolates, 12%) in a wheat field in Suffolk (sample reference 8) where soil was collected from in 2009, three years after onion was grown.

Table 6. Frequency of *Fusarium* spp. obtained from soil collected from three onion fields with onion basal rot incidence.

	Field	Fo	Fp	Fsp
1	Warwickshire (sample reference 17)	4	0	3
2		4	0	5
3		4	0	2
4		2	0	5
5		5	0	2
Total		19	0	17
1	Suffolk (sample reference 6) symptomless area	0	0	1
2		0	0	0
3		1	0	1
4		1	0	0
5		0	0	2
6	Suffolk (sample reference 6) diseased area	0	0	1
7		0	0	2
8		9	0	0
9		4	0	0
10		2	0	0
Total		17	0	7
11	Essex (sample reference 8)	1	0	3
12		0	0	2
13		1	0	3
14		0	0	4
15		0	0	3
Total		2	0	15

Fo: *F. oxysporum*, Fp*: F. proliferatum*, Fsp*: Fusarium* sp.
For sample reference number see Table 2

Intensive sampling within a single field

The same field in Suffolk (sample reference 8) was intensively sampled the following year (2010) when planted with onion. 70 onion bulbs were collected and

subjected to isolations which led to the identification of 28 *F. oxysporum* and three *F. redolens* (Table 5).

SUMMARY

British onion production is compromised by Fusarium basal rot, an increasing problem caused primarily by *F. oxysporum* f. sp. *cepae* (FOC). *F. proliferatum* causing basal rot of onion was found for the first time in three different counties in the UK. *F. oxysporum* was easily isolated from the soil of fields with a previous history of onion basal rot, even several years later, while *F. proliferatum* was only found from plant material. *F. solani* and *F. redolens* were found on onions grown in the UK as frequently (5%) as *F. proliferatum. F. avenaceum* was the least commonly found species from onion bulbs. Interestingly, isolations from greenhouse-grown leeks showing crown and basal rot symptoms led to the identification of *F. avenaceum* and *F. culmorum.* This is the first time these species have been found in the UK in diseased leeks. The presence of *Fusarium* species on imported onion sets and dry onion bulbs shows that this is one way in which pathogenic isolates can be introduced. Moreover, very aggressive FOC isolates were found from Dutch sets.

2.4.2.2 Cladistic analysis of *Fusarium* associated with *Allium* spp.

The *TEF* sequences of 197 *Fusarium* isolates, chosen based on host and origin, mainly derived from onion, but also from shallot, leek, garlic and Welsh onion from several locations were analysed by cladistics methods. Isolates clustered into seven main groups when analysed using the Neighbour-joining method (Figure 7). The seven groups were supported by high bootstrap values and corresponded to *F. oxysporum* (160 isolates), *F. proliferatum* (15 isolates), *F. solani* (13 isolates), *F.*

redolens (15 isolates), *F. culmorum* (7 isolates), *F. avenaceum* (5 isolates) and *F. acuminatum* (1 isolate).

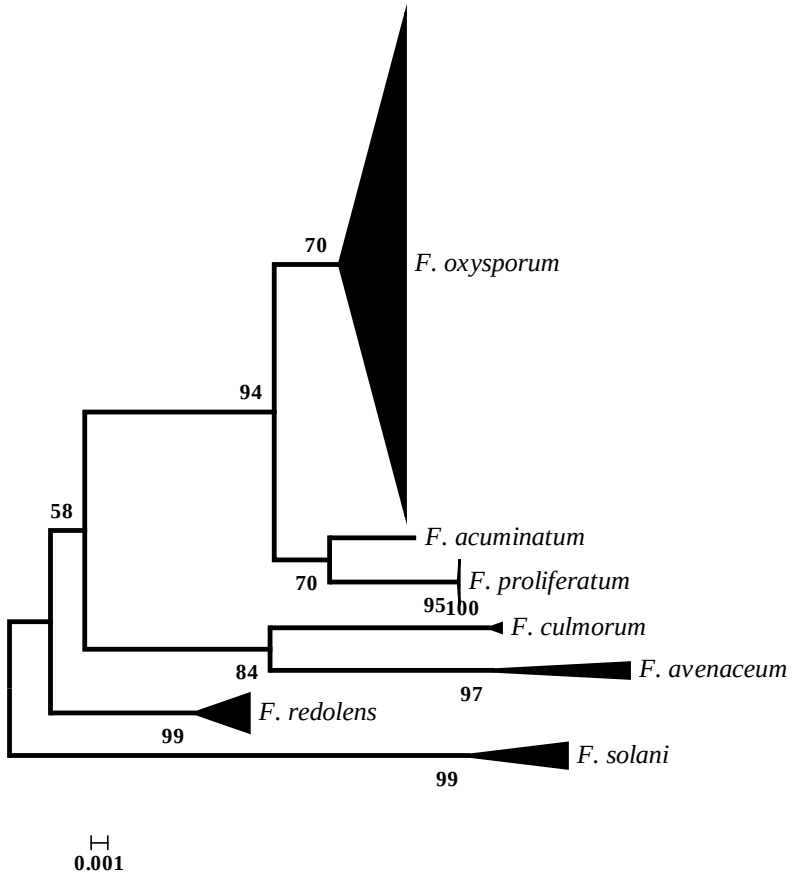


Figure 7. Cladogram of *Fusarium* species isolated from onion based on *TEF*. Consensus tree is shown with bootstrap values from 1000 replications. Triangles are representing. *Fusarium* species and the size of triangles are proportional to the number of isolates.

F. oxysporum TEF sequence variation

A UPGMA dendrogram (Figure 8) was produced comprising 47 of the 175 *F. oxysporum* isolates with *F. foetens* used as a closely related out-group. The selection of *F. oxysporum* isolates was made to represent different locations and *Allium* crops representing seven countries. To make this analysis more robust, eleven published *F. oxysporum* f. sp. *cepae* sequences deposited on NCBI were also included. *F. oxysporum* isolates associated with *Allium* species were subdivided into twelve sequence types (STs FoA – FoM), based on *TEF* sequence similarity (Kimura

distance =0.000) which was confirmed by maximum parsimony and neighbour-joining methods (data not shown). The most abundant sequence type was ST FoA with 136 isolates and ST FoB was also common with 37 isolates (Figure 8, Appendix II). The remaining sequence types were found less frequently: ST FoC (2 isolates), ST FoD (6 isolates), ST FoE (6 isolates), ST FoF (3 isolates), ST FoG (2 isolate), ST FoH (1 isolate) and ST FoI (3 isolates), ST FJ (7 isolates) and STs FoK – FoM which were represented by one isolate each (Figure 8, Appendix II).

The most common sequence types (FoA and FoB) were not associated with host or geographic origin (Appendix II). Sequence types FoA and FoB both comprised isolates from onion, garlic and leek. Moreover, isolates in ST FoA originated from Bedfordshire, Lincolnshire, Nottinghamshire, Warwickshire, Netherlands, Australia, USA and Ireland, while isolates belonging to ST FoB were recovered from Bedfordshire, Warwickshire, Suffolk, Lincolnshire, USA, Spain, New Zealand, and Italy. In addition, representatives of the two different STs were found in the same field (A25 and A28 from sample 4 in Bedfordshire, Appendix II).

A smaller group, ST FoC, comprised A5 and Fus1 isolates from onion grown in Nottinghamshire. ST FoD comprised isolates from British shallot (SH3, SH1-1) and from onions grown in Cambridgeshire (PG), Netherlands (151) and Uruguay (EU220399). Two isolates from British garlic and one *F. oxysporum* f. sp. *cepae* sequence from Germany formed ST FoE. Isolates recovered from Welsh onion from Japan (18 and 22) fell into ST FoF along with an isolate (D7-2b) recovered from Dutch onion sets. ST FoG comprised two isolates, one from onion grown in Suffolk (R3) and another from Turkey (EU220402).

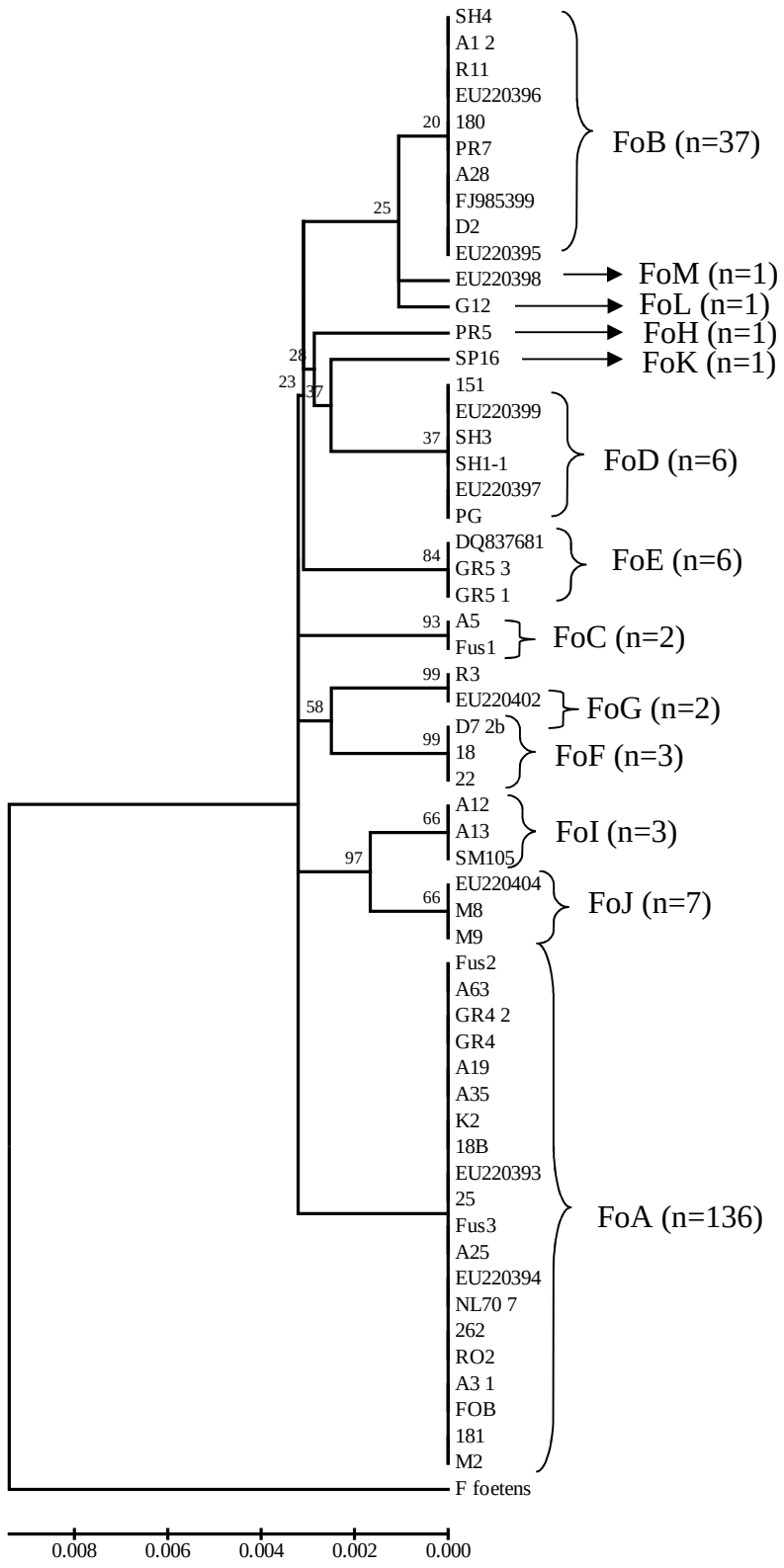


Figure 8. Dendrogram of representatives of *Fusarium oxysporum* associated with *Allium* species based on partial *TEF* dataset rooted with *F. foetens*. Consensus tree is shown with bootstrap values from 1000 replicates. GenBank accession number is indicated for each reference sequence. Twelve sequence types (FoA-FoM) were recognized. Sequence type frequencies of isolates (n) obtained during this study and used as references are shown in brackets.

A few isolates had unique STs including one of the Spanish isolates from leek (PR5, ST FoH) and another Spanish isolate from onion (SP16, ST FoK). Sequence types FoL and FoM also had only one representative each, from Dutch (EU220398) and British onions (G12) respectively.

Isolates from onion were represented in most of the ST groups such as FoA-FoE, FoG and FoI. Shallot isolates fell into ST groups FoB and FoD, while garlic isolates were represented in FoA, FoB and FoE.

The 28 *F. oxysporum* isolates from the intensively sampled field in Suffolk (sample reference 8) were identical based on the *TEF* sequence and they all fell into ST FoA. Similarly, all isolates from a field in Bedfordshire (sample reference 4), fields from Suffolk (sample references 9 and 11) and fields from Essex (sample references 5 and 6) belonged to ST FoA. One of the lower frequency sequence types, ST FoI, was recovered exclusively from a field in Bedfordshire (sample reference 3).

F. proliferatum TEF sequence variation

F. proliferatum isolates (18) derived from onion and one from garlic and an additional four published sequences which used for taxonomical reference were analysed by the neighbour-joining method (Figure 9). The *F. proliferatum* isolates from onion formed three sequence types (FP1-FP3), while the one garlic isolate belonged to FP4 (Figure 9, Appendix III). Three isolates representing FP3 originated from Spain, while FP1 and FP2 comprised isolates from more than one country. For example, eleven isolates belonging to FP1 were recovered from onions grown in Warwickshire, Lincolnshire, Ireland, Spain, USA, Chile, Netherlands and Uruguay. FP2 comprised six isolates from Warwickshire, Bedfordshire, Netherlands and

Argentina. Representatives of two sequence types were also present in the same field (e.g. A40 and A8 from sample 4 Bedfordshire, Appendix III).

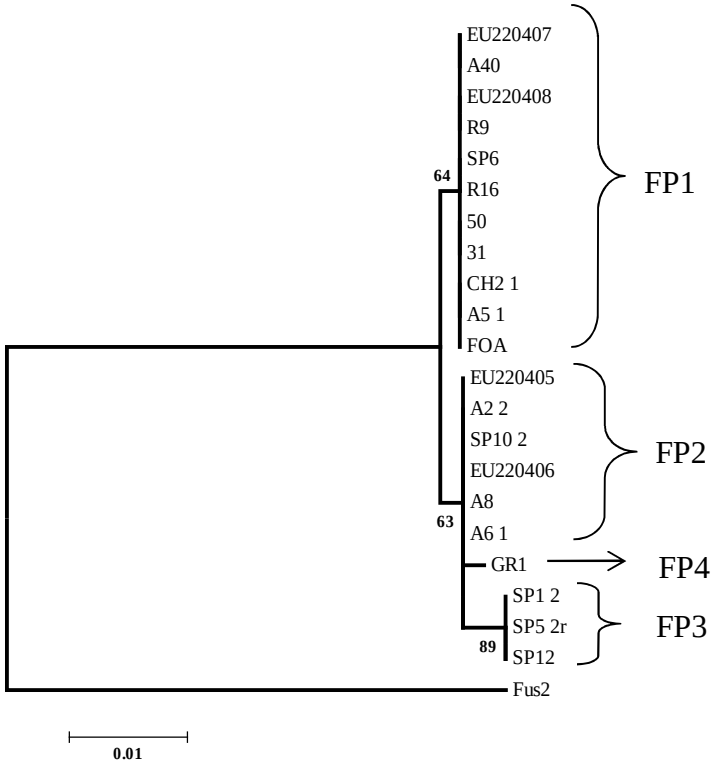


Figure 9. Cladogram of *Fusarium proliferatum* isolated from onion and garlic based on *TEF* rooted with *F. oxysporum* f. sp. *cepae* isolate Fus2. GenBank accession number is indicated for each reference sequence. Consensus tree is shown with bootstrap values from 1000 replications. Four sequence types were recognised (FP1-FP4).

F. redolens TEF sequence variation

F. redolens isolates (15) originating from Suffolk and Essex and collected from onion bulbs and soil comprised six sequence types (FR1-FR6) based on the neighbour-joining method (Figure 10, Appendix III). All isolates that originated from Essex shared the same sequence type, FR1, except NL41 (FR2). Isolates from Suffolk showed higher variation, every isolate falling into a different sequence type group.

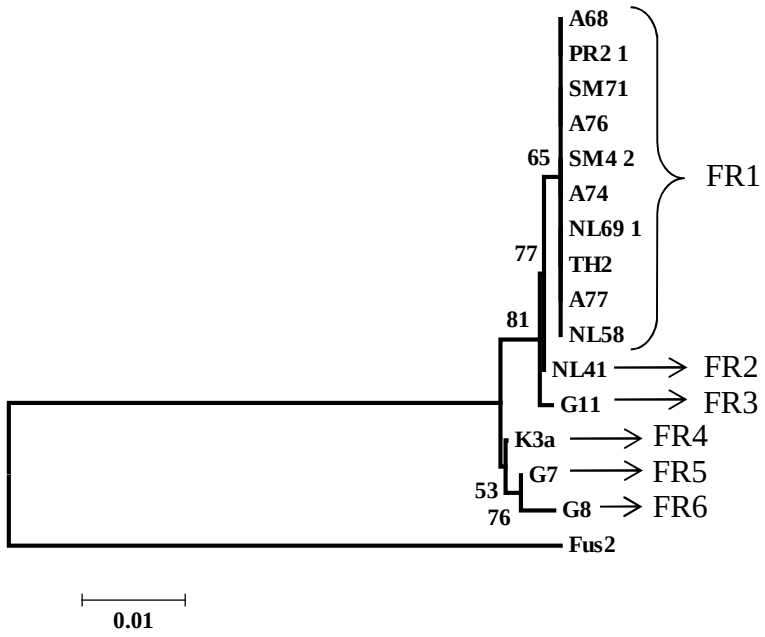


Figure 10. Cladogram of *Fusarium redolens* isolated from onion based on *TEF* rooted with *F. oxysporum* f. sp. *cepae* isolate Fus2. Consensus tree is shown with bootstrap values from 1000 replications. Six sequence types were recognised (FR1-FR6).

F. solani TEF sequence variation

F. solani isolates (13) analysed by the neighbour joining method based on *TEF* sequence (Figure 11, Appendix III) fell into seven sequence type groups (FS1-FS7). Fus2, a *F. oxysporum* isolate was used as out-group. *F. solani* isolates were collected from onion, garlic and soil and originated from Suffolk, Essex, Bedfordshire and Lincolnshire. The only garlic isolate from Bedfordshire (GR5_4)

formed a distinct sequence type (FS4). FS1 comprised isolates from Suffolk and Essex both from onion and soil, while isolates belonging to FS6 originated from Essex, Lincolnshire and Bedfordshire. The remaining isolates formed individual sequence types.

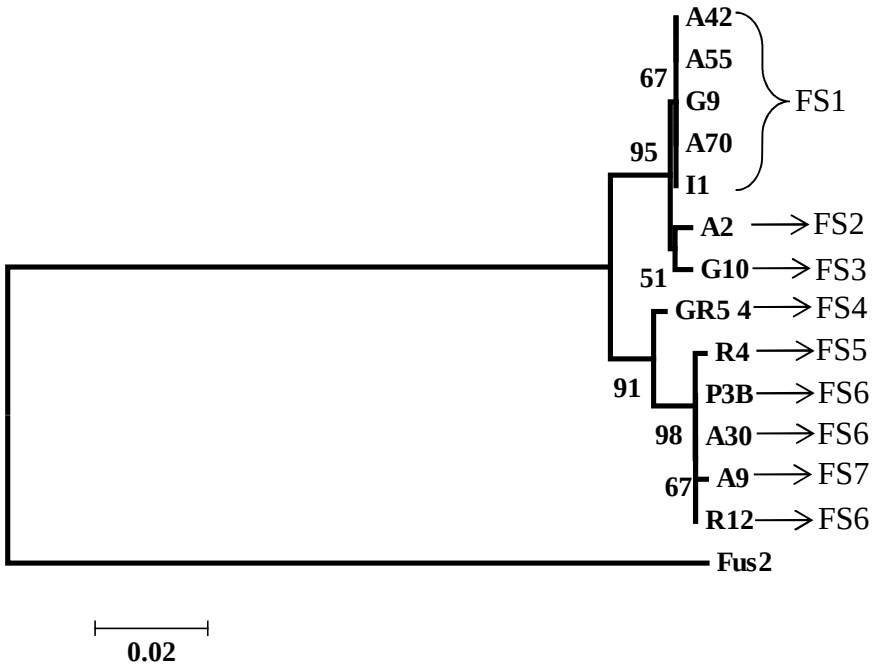


Figure 11. Cladogram of *Fusarium solani* isolated from onion and garlic based on *TEF* rooted with *F. oxysporum* f. sp. *cepae* isolate Fus2. Consensus tree is shown with bootstrap values from 1000 replications. Seven sequence types were recognised (FS1-FS7).

F. avenaceum, F. culmorum and *F. equiseti TEF* sequence variation

Six *F. avenaceum* isolates from British onion and leek with one additional sequence used as taxonomic reference (EU220414) were analysed based on *TEF* sequences using the neighbour joining method (Figure 12, Appendix III). Two isolates derived from leeks grown in Cambridgeshire were identical and formed sequence type FA1. The rest of the isolates were different and fell into individual sequence types (FA2 – FA6). *F. culmorum* isolates obtained from leek and soil all had identical *TEF* sequences so formed only one ST (Appendix III). Similarly, three

F. equiseti isolates from the soil of one particular field again formed one sequence type group (Appendix III).

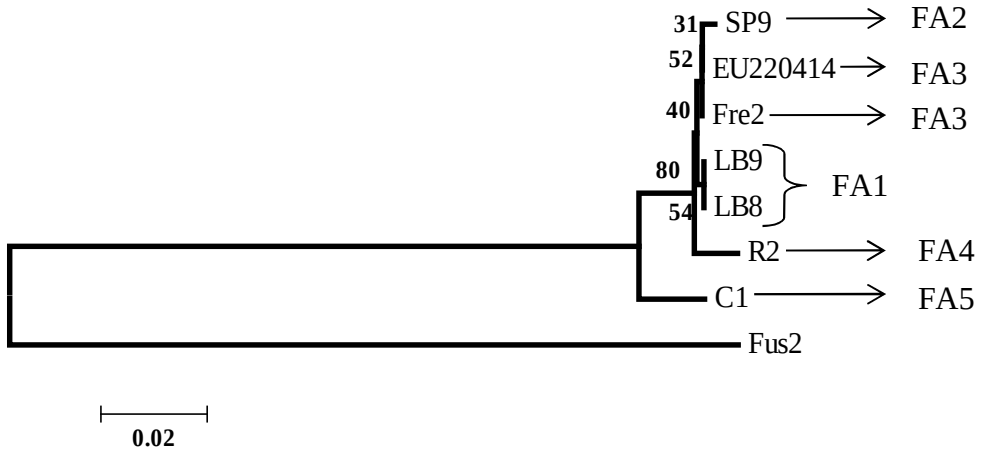


Figure 12. Cladogram of *Fusarium avenaceum* isolated from onion and leek based on *TEF* rooted with *F. oxysporum* f. sp. *cepae* isolate Fus2. GenBank accession number is indicated for each reference sequence. Consensus tree is shown with bootstrap values from 1000 replications. Four sequence types were recognised (FA).

SUMMARY

The *TEF* sequences of 197 *Fusarium* isolates from onion, shallot, leek, garlic and Welsh onion from several locations were analysed by cladistics methods. Isolates clustered into seven main groups supported by high bootstrap values and corresponded to *F. oxysporum* (160 isolates), *F. proliferatum* (15 isolates), *F. solani* (13 isolates), *F. redolens* (15 isolates), *F. culmorum* (7 isolates), *F. avenaceum* (5 isolates) and *F. acuminatum* (1 isolate). Representatives of each of these species were further analysed by comparing their *TEF* sequences to previously published sequences of *Fusarium* isolates associated with onion basal rot. Results of the sequence analysis of *TEF* suggest that *F. oxysporum* associated with onion basal rot in the UK is composed of at least nine different sequence types (STs FoA-FoL). The *F. proliferatum* isolates from onion formed three sequence types (FP1-FP3), while

the one garlic isolate belonged to FP4. *F. redolens* isolates (15) comprised six sequence types (FR1-FR6), *F. solani* isolates (13) seven sequence types (FS1-FS7), and *F. avenaceum* (5) five sequence types (FA1-FA5). *F. culmorum* (7) isolates did not show any *TEF* sequence variation. In general, sequence types were not associated with host or geographic origin.

2.5 DISCUSSION

2.5.1 Testing and development of primer pairs used for *Fusarium* identification

Identification of fungal species is traditionally based on morphological characteristics. Identification of *Fusarium* species based on morphology is reliable if done by well-trained staff, but the recent development of molecular methods provides the opportunity for a quicker and more reliable identification of *Fusarium* by researchers without any prior knowledge of *Fusarium* taxonomy. The other great advantage of molecular methods is that they do not necessarily require pure cultures as infected plant samples can also be used. *TEF* is the most widely used molecular marker for *Fusarium* species identification and ideally requires sequence comparison with type isolates (Geiser *et al.*, 2004; Park *et al.*, 2010). In some cases, e.g. *Fusarium graminearum* species complex, multi-locus genotyping is required for species-level identification (Ward *et al.*, 2008). Molecular methods are expensive and therefore cost effective DNA extraction methods and the use of *Fusarium* genus- and species-specific primers were tested.

The 10% Chelex 100 DNA extraction method was suitable for PCR-based analysis, including amplification of single-copy targets (e.g. *TEF*). It is a quicker and more cost effective way of DNA extraction compared to the commercially available kits (from Qiagen and Sigma), but gave poorer quality and lower amounts of DNA.

Two *Fusarium* genus-specific primer pairs based on the ITS region of the rDNA published in the literature were tested. The *Fusarium*-specific primer pair (FU-ITSf/FU-ITSr) published by Bluhm *et al*. (2004) was more reliable than the sequences (ITS-FUf/ITS-Fur) designed by Abd-Elsalam *et al*. (2003). Only *F.*

oxysporum, F. solani, F. verticillioides and *Rhizoctonia solani* sequences were used by Abd-Elsalam *et al.* to test primer design, while Bluhm *et al.* utilised nine *Aspergillus* spp., nine *Fusarium* spp., and ten *Penicillium* spp. sequences. Differences in the number of species representing different genera used to test primer pairs could explain the differences in specificity.

F. oxysporum and *F. proliferatum*-specific PCRs (CLOX1/CLOX2 and CLPRO1/CLPRO2; Mule *et al.,* 2004) were potentially suitable for distinguishing these two species based on presence / absence of amplicons, but the representatives of other *Fusarium* species had to be identified based on *TEF* sequencing.

Newly developed *Fusarium* genus-specific primers based on *TEF* were designed to accelerate the identification of *Fusarium* isolates (exTEFfor and FUexTEFrev). Initially, the specificity of these primers was tested *in silico* on 12 fungal species, including four *Fusarium* and eight closely related species. These were also successfully tested *in vitro* on 20 *Fusarium* species and a few closely related species (*M. nivale* and *C. gloeosporioides*) and proved to be a rapid and reliable method for identifying *Fusarium* species associated with *Allium* crops. During this three-year-project about 300 *Fusarium* isolates were identified using the newly developed primer pair and the identity about 200 of these was confirmed by sequencing the *TEF* gene. All of these sequences belonged to the representatives of *Fusarium* genus. Nevertheless, the robustness of identification using newly developed primer pair (exTEFfor and FUexTEFrev) could be enhanced by testing its specificity *in vitro* on several fungal genera.

Recent publications on onion basal rot have reported the use of morphological identification and combined with *Fusarium* species-specific primers but isolates belonging to *F. solani* had to be identified based on *TEF* sequencing

(Bayraktar *et al*., 2010; Galvan *et al*., 2008). Sequences of primer pairs applicable for the identification of *F. solani* and the *Fusarium* genus based on *TEF* have only been published earlier this year (Arif *et al*., 2012).

2.5.2 Genetic diversity of *Fusarium* species associated with *Allium* species in the UK

Onion basal rot incidence came to the attention of British growers and agronomists recently, because there has been a considerable increase in disease levels in the last few years (A. Richardson, Allium and Brassica Centre pers. comm.). This is the first comprehensive study which has investigated the identity of pathogens causing onion basal rot in the UK as well as their genetic variation.

F. oxysporum was the most commonly isolated species from onion bulbs collected between the 2008 and 2010 in the UK and this is consistent with previous findings from the Netherlands and Turkey (Bayraktar *et al*., 2011; Galvan *et al*., 2008). *F. proliferatum* was found for the first time in the UK in onions with basal rot symptoms collected in three different counties. *F. proliferatum* has been identified as a causal agent of basal rot in other countries (Galvan *et al*., 2008, du Toit *et al.*, 2003, Stankovic *et al*., 2007). *F. proliferatum* infecting onion, may affect the health safety of agricultural workers as well as consumers. Fumonisin B1, beauvericin, fusaric acid, fusaproliferin and moniliformin production of *F. proliferatum* stains isolated from onion and garlic were confirmed by Stankovic *et al.* (2007) and Seefelder *et al*. (2002). Fumonisin B1 has been linked to human cancer and mycotoxicosis (Gelderblom *et al*., 1991). Beauvericin, fusaproliferin, moniliformin and fusaric acid has been found toxic to various human cell lines (Logrieco *et al*., 2002; Logrieco *et al*., 1996; Celik *et al*., 2009), Additionally, Fumonisin B1was found to cause chromosomal aberrations in onion cells (Lerda *et al*., 2005). Moreover, all these toxins are considered as phytotoxins, therefore suspected to be involved in the expression of symptoms on onion (Stankovic *et al*., 2007). However, further studies

have to be carried out in terms of *in vitro* and *in planta* toxin production to evaluate risk to plant and human health.

F. solani and *F. redolens* were found on onions grown in the UK as frequently as *F. proliferatum*. Both species were reported as pathogens of onion in Turkey, and Serbia (Bayraktar *et al*., 2010; Klokocar-Smit *et al*., 2008). *F. avenaceum* was the least commonly found species from British grown onion bulbs which agrees with previous findings from the Netherlands (Galvan *et al.*, 2008). A few *F. equiseti* isolates were recovered from the soil of one of the fields in Essex. *F. equiseti* is a common soil saprotroph and also can be associated with head blight of cereals (Nicholson *et al*., 2003).

Interestingly, isolations from greenhouse-grown leek showing basal rot symptoms led to the identification of *F. culmorum* and *F. avenaceum*. This is the first time these species have been found in the UK in leek with crown and basal rot symptoms. *F. culmorum* was previously identified as the causal agent of basal rot of leek in Spain and California (Armengol *et al*., 2001; Koike *et al*., 2003) and was also reported as an onion pathogen in the Netherlands (Galvan *et al*., 2008). *F. avenaceum* has been identified as the causal agent of crown rot of leek (Hall *et al*. 2007). Moreover, both species are associated with Fusarium head blight of cereals in the UK (Nicholson *et al*., 2003). Although, *F. oxysporum* was not found in diseased greenhouse-grown British leeks, it has been recently isolated from field-grown leek showing basal rot symptoms (Andrew Taylor, University of Warwick, pers. comm.).

F. oxysporum was also present on shallot and garlic showing basal rot symptoms in the UK. These results agree with previous publications (Sintayehu *et al*., 2011, Palmero *et al*., 2012.). Additionally, *F. proliferatum* and *F. solani* were also isolated from garlic. *F. proliferatum* was confirmed as causal agent of basal rot

of garlic in Spain, Serbia, USA, China and Italy (Palmero *et al*., 2012, Stankovic *et al*., 2007, Dugan *et al*., 2007; Tonti *et al*., 2012). *F. solani* has not been reported from garlic before, although its pathogenic ability has yet to be confirmed.

The presence of *Fusarium* species on imported onion sets and dry onion bulbs shows that this is one way in which pathogenic isolates can be introduced. *F. oxysporum* was found from Dutch sets showing basal rot symptoms and on symptomless Belgian import sets. The fields where the Dutch and Belgian sets were planted suffered from onion basal rot although the disease had not been reported in the area previously (A. Richardson, A & B Centre, pers. comm.). These results suggest that new outbreaks of *Fusarium* might be caused by the import of diseased onion sets, and hence suggest that closer inspection of such planting material is required. Spanish, Chilean and Dutch imported onion bulbs showing basal rot symptoms were mainly infected by *F. oxysporum*. Moreover, *F. proliferatum* was recovered from Spanish and Chilean imported onion bulbs. Almost half of the isolates from the Spanish sample belonged to *F. proliferatum* and the two less common species, *F. acuminatum* and *F. avenaceum*, were also present. Most diseased onions are discarded during the grading process in the storage facilities, but some symptomless bulbs may develop disease during transport and storage.

F. oxysporum was easily isolated from the soil of fields with a previous history of onion basal rot, even several years later. A high proportion of the isolates obtained from a severely diseased part of a field in Suffolk were identified as *F. oxysporum*. More than 50% of isolates collected from a field in Warwickshire (five months after harvest) were identified as *F. oxysporum*. It was also present in soil from a field in Essex where onions had not been grown for 3 years. This same field was sampled intensively for decaying bulbs the following year and had a very high

level of basal rot. This suggests that the level of propagules is very high by the end of the onion growing season and that the pathogen does survive several years in soil. This is not unexpected as the decaying bulbs are not collected (pers. observation) and removed from the fields and the grower only uses a 4-year-rotation. *F. oxysporum* can survive on soil debris as mycelium or as chlamydospores (Abawi and Lorbeer, 1972). Interestingly, *F. proliferatum* was only found from plant material, which could be due to lack of chlamydospore production (Leslie and Summerrell, 2006) or because *F. proliferatum* mainly causes disease in storage. A few other isolates were also identified as *F. equiseti, F. culmorum* and *F. redolens* which commonly occur in soils. *F. equiseti* and *F. culmorum* are also associated with head blight of wheat which is grown in rotation with onion (O`Connor, 2005).

Results of the sequence analysis of *TEF* suggest that *F. oxysporum* isolates associated with onion basal rot in the UK are composed of at least nine different sequence types (STs), suggesting that pathogenic ability may have evolved convergently. Transfer of pathogenicity-related genes of *F. oxysporum* f. sp. *lycopersici* by parasexuality and horizontal gene transfer have been proposed (Ma *et al*. 2010). Several VCG groups have been identified within FOC (Swift *et al*., 2002; Southwood *et al*., 2012) which also indicates genetic variation. FOC isolates have different levels of aggressiveness which suggests that lineages may also have different pathogenic properties (Taylor *et al*., 2012). Moreover, the *TEF* sequence types of FOC cannot be explained by the current geographic distribution. It was not uncommon to recover only one sequence type from one particular field. For instance, all *F. oxysporum* isolates recovered from the intensively sampled field in Essex belonged to the same sequence type “FoA” suggesting a clonal origin. This lack of

geographic association may due to human activity *e.g.* global trade of planting material and therefore the spread of onion basal rot (Galvan *et al*., 2008).

During this study six *Fusarium* species were identified on diseased *Allium* crops in the UK, namely *F. oxysporum, F. proliferatum, F. solani, F. redolens, F. culmorum* and *F. avenaceum.* The role of these species needed to be defined (e.g. pathogen, facultative pathogen and saprophyte), in order to manage diseases and to develop methods applicable for identification of pathogenic forms. The pathogenicity of these different species on onion is examined in Chapter 3.

CHAPTER 3

MOLECULAR IDENTIFICATION AND CHARACTERISATION OF *FUSARIUM OXYSPORUM* FORMAE SPECIALES

3.1 INTRODUCTION

3.1.1 Molecular techniques used for characterisation of *F. oxysporum* pathogen populations

The use of various molecular methods in order to distinguish *F. oxysporum* formae speciales (ff. spp.) has led to the same conclusion: most formae speciales are polyphyletic, but with a few exceptions (Leslie and Summerell, 2006). This section gives a short summary of the various methods and genes used over the last twenty years, which led to above conclusion.

Restriction fragment length polymorphisms (RFLP) is used for the analysis of populations as genomic DNA of different isolates may result in a distinct pattern after digestion with various restriction enzymes and separation by electrophoresis. Variations are generated by mutations that create or abolish recognition sites for these enzymes or change the length of the fragment (NCBI). RFLP gives information on genetic diversity by covering the whole genome and is reproducible but requires large amounts of DNA. *F. oxysporum* ff. spp. *cubense*, *dianthi*, *gladioli*, *lycopersici*, *niveum,* and *vasinfectum* were found to be polyphyletic while *F. oxysporum* f. sp. *albedinis* was monophyletic based on their RFLP pattern (Elias *et al*., 1993; Fernandez *et al*., 1998; Kim *et al*., 1992, Manicom and Baayen; 1993; Koenig *et al*, 1997; Mes *et al.*, 1994; Tantaoui *et al*, 1996).

Random amplified polymorphic DNA (RAPD) markers are PCR amplicons of random segments of genomic DNA with a short single primer (Williams *et al*., 1990). RAPD is a cheap and powerful method for the characterization of species, but it can be difficult to reproduce the same results (*i.e*. laboratory dependent). Specific bands can be identified based on amplicon-pattern and be used to develop sequenced characterized amplified region markers (SCARs). Using RAPD markers, *F.*

oxysporum ff. spp. *batatas, cubense, cucumerinum, dianthi, gladiolus, nicotianae, phaseoli, pisi,* and *radicis-cucumerinum* were found to be polyphyletic while *F. oxysporum* f. sp. *ciceris* was described as a monophyletic f. sp. (Alves-Santos *et al*., 2002; Bentley *et al*., 1995; Clark *et al*., 1998; Haan *et al*., 2000; Grajal-Martin *et al*., 1993; Wright *et al*., 1996; Lievens *et al*., 2007; Manulis *et al*., 1993, Vakalounakis *et al*., 2004; Jiménez-Gasco *et al*., 2002).

A less frequently used fingerprinting method is AFLP analysis, which involves the fragmentation of total genomic DNA and the selective amplification of fragments (Vos *et al*. 1995). The advantage of using this method is that it covers the whole genome and is reproducible. Conversely, it is more expensive and time consuming. The monophyly of *F. oxysporum* ff. spp. *lilii* and *tulipae* were supported by AFLP profiles, but *F. oxysporum* ff. spp. *asparagi, cepae, cubense, dianthi, gladioli, lini, opuntiarum* and *spinaciae* were found to be polyphyletic (Baayen *et al*., 2000; Galvan *et al*., 2008; Groenewald *et al*., 2006).

Multi-locus sequence typing (MLST) offers a robust approach for characterizing the genetic diversity in the *F. oxysporum* species complex (O'Donnell *et al*., 2009).

Translation elongation factor 1α subunit (*TEF*) and intergenic spacer of rDNA (*IGS*) have been found to be highly informative markers which are sensitive to evolutionary change. A two-locus DNA database was constructed based on partial *TEF* and *IGS* sequences of 850 *F. oxysporum* isolates representing various formae speciales (O`Donnell *et al*., 2009). Nearly 300 (256) two-locus sequence types were differentiated and the dataset suggested that two-thirds of them may be associated with a single host plant.

Microsatellites (simple sequence repeats, SSR) are 2-6 base pair sequence repeats which can vary among isolates of the same species or related species and therefore can be used as molecular markers. Microsatellites were employed to study genetic diversity of *F. oxysporum* ff. spp. *lycopersici, radicis-lycopersici, ciceris* and soil isolates (Barve *et al*., 2001; Bayraktar *et al*., 2008; Jimenez-Gasco *et al*., 2004; Balmas *et al*., 2005; Bogale *et al*., 2006). Analysis of these data revealed the stepwise evolution of eight races of *F. oxysporum* f. sp. *ciceris* when multiple gene genealogies failed due to lack of polymorphism (Jimenez-Gasco *et al*., 2004).

3.1.4 Specific identification of *F. oxysporum* formae speciales

Members of the *F. oxysporum* species complex have been characterised using biotypic and molecular methods in an attempt to identify agriculturally important pathogenic isolates.

Pathogenicity assays provide a robust solution for distinguishing various formae speciales, but this involves the infection of more than a hundred plant species which is very time consuming and laborious (Leslie and Summerell, 2006).

Vegetative compatibility provides another widely used biotypic characterisation method that is based on the ability of isolates to anastomose and form heterokaryons within vegetative compatibility groups (VCGs) (Katan, 1999). A worldwide collection of *F. oxysporum* f. sp. *lycopersici* isolates was characterized by the VCG method and molecular methods subsequently revealed that isolates within each VCG are clonal derivatives of a common ancestor. Therefore, VCGs are reliable indicators of evolutionary origin and appear to be clonal lineages (Elias *et al*., 1993).

Molecular variation in populations of *F. oxysporum* ff. spp. *cucumerinum*, *radicis-cucumerinum, ciceris* (races 0, 1A, 5, and 6), *dianthi* (race 1), *basilica* and *phaseoli* was analysed to develop PCR-based markers (RAPD and more specific SCAR markers) for distinguishing amongst formae speciales and races (Lievens *et al*., 2007; García-Pedrajas *et al*., 1999; De Haan *et al*., 2000; Chiocchetti *et al*., 2001; Alves-Santos *et al*., 2002; Jiménez-Gasco and Jiménez-Díaz, 2003; Kelly *et al*., 1998; Pasquali *et al*., 2006).

Transposable elements can also be used for the specific identification of various formae speciales. *F. oxysporum* f. sp. *albedinis*, a pathogen of date palm, and *F. oxysporum* f. sp. *chrysanthemi*, a pathogen of Paris Daisy were specifically detected based on the differences in the transposable element, *Fot1* sequence (Fernandez *et al*., 1998; Pasquali *et al*., 2004a; Pasquali *et al*., 2004b). Primers designed to amplify *Fot1* and *impala* elements allowed the differentiation of *F. oxysporum* f. sp. *dianthi* races 1, 2, 4, and 8 (Chiocchetti *et al*., 1999). *F. oxysporum* f. sp. *lactucae* race 1 and *F. oxysporum* f. sp. *cepae* (FOC) VCG 0425 and 0421 isolates were detected by an inter-retrotransposon sequence-characterized amplified region technique which employed primers designed on long terminal repeats, *Skippy* and *Han*-solo (Pasquali *et al*., 2007; Southwood *et al*., 2012).

Housekeeping genes are useful for investigating the phylogeny of fungi, however, this seldom correlates with the pathogenic ability of plant pathogens, therefore in these cases housekeeping genes can be used to develop specific identification / detection methods (Talhinhas *et al*., 2002; Barbara and Clewes, 2003). For example, *Colletotrichum acutatum* isolates causing anthracnose on lupin form a distinct clade, based on ITS sequence, from other pathogenic forms within this species (Talhinhas *et al*., 2002).

Based on single nucleotide polymorphisms present in the *IGS* region, PCR diagnostic tools were developed to detect the *F. oxysporum* f. sp. *cubense* tropical race 4 (Dita *et al*., 2010).

Metabolic enzymes have been used to distinguish variation in *F. oxysporum* f. sp. *lycopersici* and *radicis-lycopersici* and races of *F. oxysporum* f. sp. *lycopersici* originating from Japan, including endo-polygalacturonase (*PG1*) and exo-polygalacturonase (*PGX4*) sequences (Hirano and Arie, 2006).

And finally, effector genes are beginning to provide a useful means for identifying *F. oxysporum* formae speciales and races (Rep *et al*, 2004; Lievens *et al*., 2009ab). For instance, specific detection of *F. oxysporum* ff. spp. *lycopersici* (races 1-3) and *vasinfectum* isolates was achieved based on the presence and sequence polymorphism of the *SIX* (secreted in xylem) and *PEP* (putative effector protein; Lievens *et al*., 2009a; Chakrabarti *et al*., 2011). *F. oxysporum* isolates have been previously screened for the presence of seven *SIX* genes encoded on a mobile pathogenicity chromosome (MPC) (Lievens *et al*., 2009a; Ma *et al*., 2010); *SIX1-SIX5* were only found in *F. oxysporum* f. sp. *lycopersici* isolates, *SIX6* and *SIX7* were present in a few other formae speciales, such as *melonis, lilii* and *racicis-cucumerinum.* A recent study on *F. oxysporum* f. sp. *cubense* reported the presence of homologues of *SIX1*, *SIX7* and *SIX8* (Meldrum *et al*., 2012). Homologues of *SIX1, SIX4, SIX8* and *SIX9* were identified in a *F. oxysporum* strain infecting *Arabidopsis thaliana* (Thatcher *et al*., 2012). Another highly expressed gene, *Fusarium* transcription factor 1 (*FTF1*), was identified during the infection of *F. oxysporum* f. sp. *phaseoli*, and it is also present in *F. oxysporum* f. sp. *lycopersici* (Ramos *et al*., 2007).

3.2 AIMS AND OBJECTIVES

The specific objectives of this chapter were:

1. To test the pathogenicity of different isolates of *Fusarium* species derived from *Allium* crops and of representatives of different formae speciales in an onion seedling bioassay.

2. To characterise *F. oxysporum* f. sp. *cepae* (FOC) isolates and other formae speciales based on housekeeping and effector gene sequences and microsatellites.

3. To compare the results of the pathogenicity assay with multigene sequence and microsatellite data.

3.3 MATERIALS AND METHOD

This Chapter describes the molecular methods and onion seedling assay used for the characterisation of *Fusarium* isolates (Figure 13). *Fusarium* isolates from diseased *Allium* species were previously characterised based on *TEF* sequences (Chapter 2). In this chapter, using the *TEF* sequences of these *F. oxysporum* isolates additional isolates representing several formae speciales were identified as identical based on their *TEF* sequence similarity.

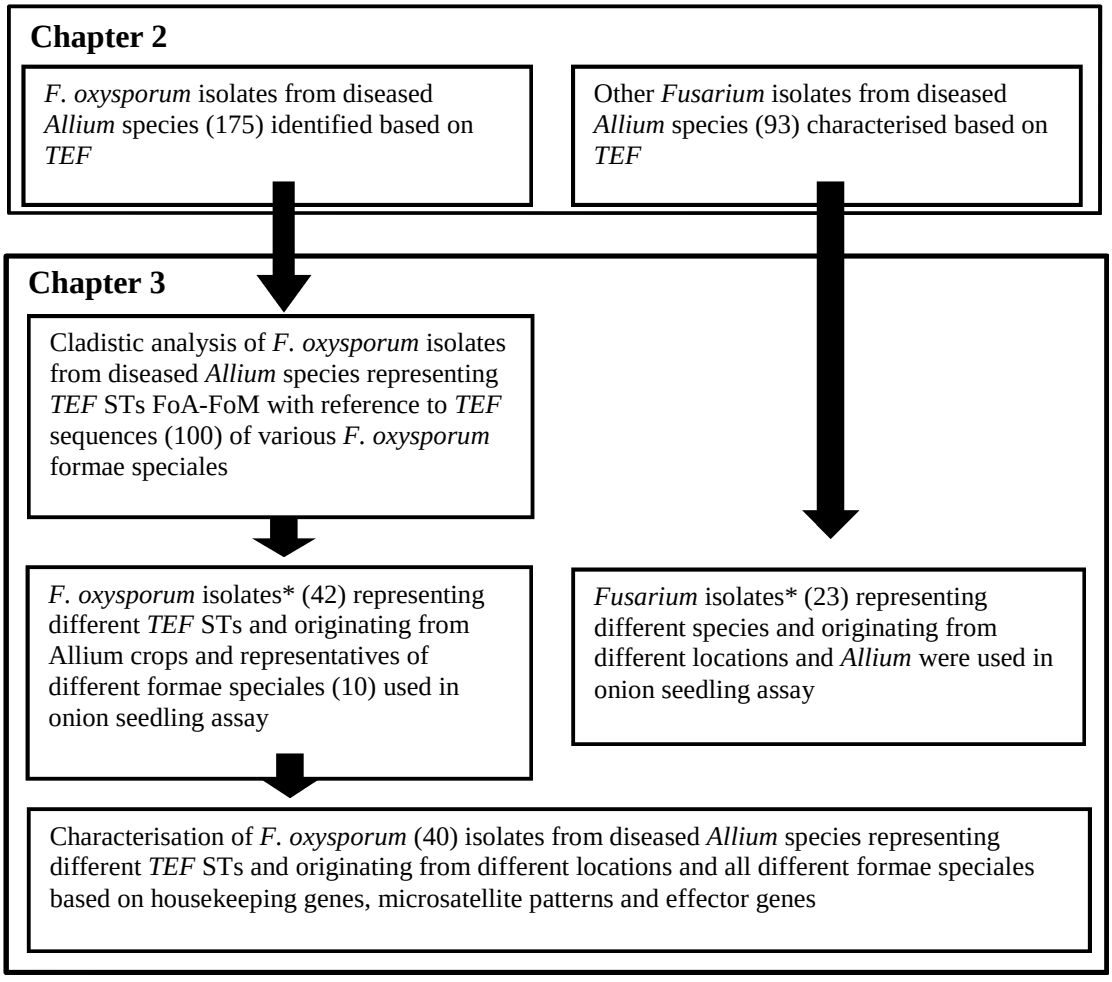


Figure 13. Flow-chart of steps used for the characterisation of *Fusarium* isolates from diseased *Allium* species.

Following this, representatives of *Fusarium* species associated with basal rot symptoms and representatives of several different formae speciales were then used in an onion seedling assay to determine aggressivess. Based on combined results of *TEF* sequence data and the onion seedling assay, *F. oxysporum* isolates were then chosen to be further characterised based on sequences of housekeeping genes, microsatellites and effectors (Figure 13).

3.3.1 Cladistic analysis of *F. oxysporum* isolates associated with diseased *Allium* species and different formae speciales based on *TEF* sequences

To initially define how *F. oxysporum* isolates obtained from diseased *Allium* species related to the three clades of the *F. oxysporum* species complex published by O'Donnell *et al*. (1998a; Figure 1), 28 *TEF* sequences of *F. oxysporum* isolates associated with diseased *Allium* species along with 35 sequences used in the research paper mentioned above were analysed using maximum likelihood method (Felsenstein, 1981) by running PAUP* b10 (Swofford, 1997) interference from inside Geneious^{TM} Pro v5.4 (Drummond *et al*., 2011).

A second method, cladistics, was also used to analyse genetic relationships among *F. oxysporum* isolates from diseased *Allium* species within the *F. oxysporum* species complex. *TEF* sequences of *F. oxysporum* isolates from diseased *Allium* species representing *TEF* STs FoA-FoM (see Chapter 2, Appendix II) along with sequence data of 100 representatives of 39 formae speciales published by O'Donnell *et al*., (1998), Baayen *et al*. (2000), Wunsch *et al*. (2009) and Galvan *et al*. (2008) were analysed by the neighbour-joining method (Saitou and Nei, 1987) using MEGA 4.0 (Tamura *et al*., 2007). To assess the topology of branches, bootstrap (Felsenstein, 1985) analysis was performed with 1000 replicates.

3.3.2 Onion seedling assay of *Fusarium* isolates from diseased *Allium* species

The aggressivess of 52 *Fusarium* isolates was tested on onion cv. Hystar F1 (Elsoms, UK) using a seedling test described by Taylor *et al*. (2012). Isolates were grown on PDA medium for ten days at 22°C at 12 hours light/dark cycle. Sterile distilled water (9 ml) was pipetted onto each plate and spores were released by using an L-shape spreader. The suspensions were filtered separately through sterile glass wool to remove hyphal fragments. Spores were counted in a haemocytometer and diluted in sterile distilled water to 1 x 10^6 spores/ml in a final volume of 5 ml. Onion seeds (112) were inoculated by incubation for one hour in the spore suspension. Onion seeds soaked in sterile distilled water were used as control. Fus2, a very aggressive FOC isolate, was used as positive standard and a *F. oxysporum* isolate Fo47 (a non-pathogenic biocontrol agent; Alabouvette *et al*., 2007), as a non-aggressive control (negative standard). Fifteen *Fusarium* isolate treatments were set up per experiment including the Fus2 and water treatment (Appendix IV). For each treatment, inoculated onion seeds (112) were sown individually into four 4x7-well-trays containing Levingtons F2 compost (Scotts) and grown at 25°C day and 18°C night under cycles of 16 hours light/8 hours dark. Trays were arranged in a randomized block design within a glasshouse compartment over two benches (Figure 14). Trays were watered through matting for four weeks and at the end of this period the numbers of surviving seedlings were recorded. A survival index was calculated for each treatment in a residual maximum likelihood (REML) procedure for linear mixed models on angular-transformed data with GENSTAT v. 13 (VSN International). The REML procedure was chosen over ANOVA to include row and column effects, because the seedling assay design was unbalanced. The model for the

analysis consisted of random factors (date, row and column) and a fixed factor (treatment).

Isolates significantly not different (l.s.d. 5%) from Fus2 (positive control) were considered very aggressive. Isolates significantly different from both Fus2 and Fo47 (negative control) were considered as moderately aggressive, while those not significantly different from Fo47 were considered as non-aggressive. Highly and moderately aggressive isolates were termed *F. oxysporum* f. sp. *cepae*.

In total *F. oxysporum* and other species isolates (74) from diseased onion, leek, shallot and garlic were chosen based on their *TEF* sequences to be tested in onion seedling assay (Table 7). Additionally, ten *F. oxysporum* isolates representing several different formae speciales were also included (Table 7).

Additionally, 14 *F. oxysporum* isolates (Fus2, 180, D2, NL96, A13, Fus1, A5, FOA5, Fom004, NRRL 36425, NRRL 26988, NRRL 26222, NRRL 36311 and Fo47) were tested using the same protocol as before, except the final spore concentrations were 2 x 10^4 spores / ml.

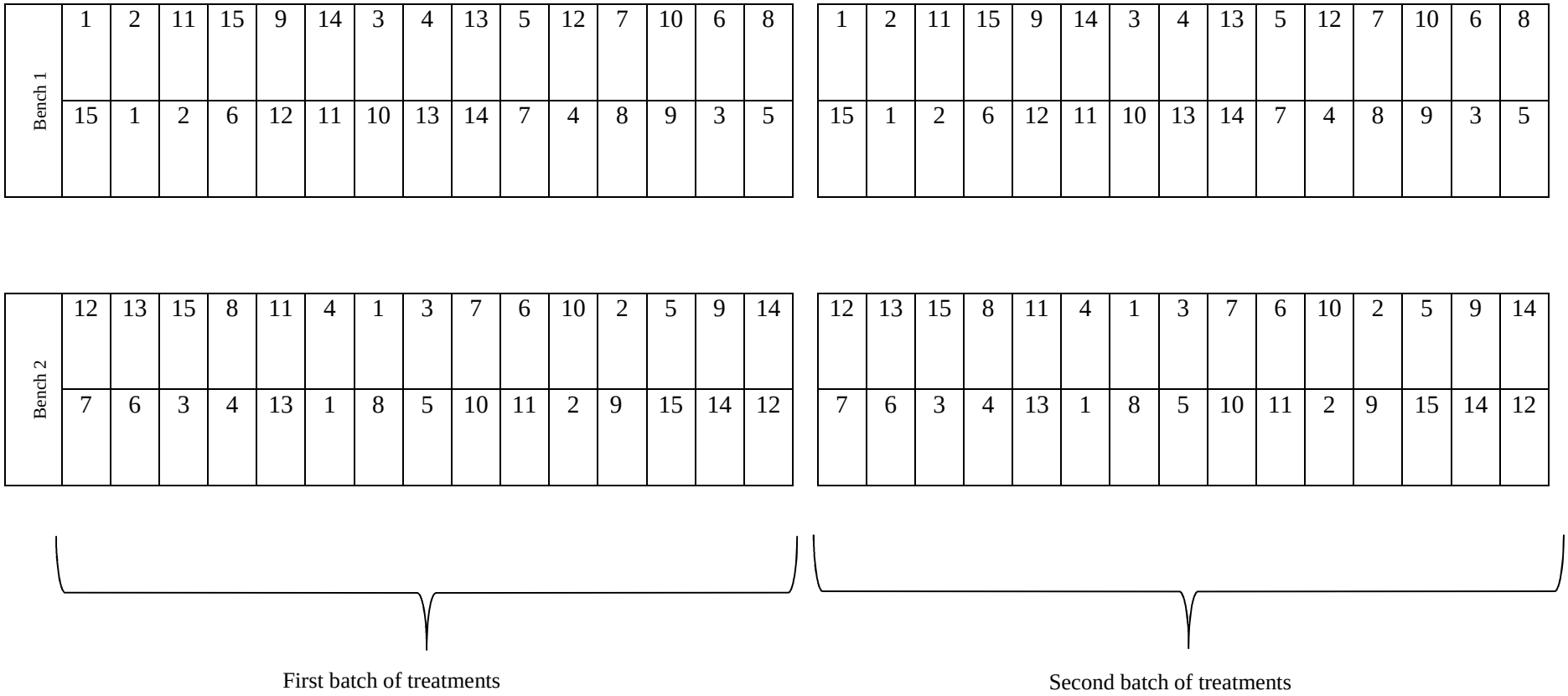


Figure 14. Seedling assay for determining levels of *Fusarium* pathogenicity (randomised block design). 15 treatments were tested and each treatment was repeated four times. Each square represents a 4x7 well-seedling tray. One seed was planted into each well (*i. e.* 28 seeds per treatment).

Table 7. Host and species level distribution of *Fusarium* isolates (74) used in onion seedling assay. Forty *F. oxysporum* isolates (**in bold**) were chosen for characterisation based on housekeeping genes, microsatellite markers and effectors.

Isolate	Species	Host	Isolate	Species	Host
22	FO *cepae*	Welsh onion	**SM54**	FO	onion
25	FO *cepae*	onion	SP14	FO	onion
151	FO	onion	SP2	FO	onion
180	FO	onion	SP7_2	FO	onion
262	FO	onion	31	FP	onion
18B	FO	leek	50	FP	onion
A1/2	FO	onion	A40	FP	onion
A13	FO	onion	A6/1	FP	onion
A14	FO	onion	A8	FP	onion
A19	FO	onion	A6/1	FP	onion
A28	FO	onion	A8	FP	onion
A35	FO	onion	A6/1	FP	onion
A5	FO	onion	FOA	FP	onion
CH1_1	FO	onion	R16	FP	onion
D2	FO	onion	R9	FP	onion
FOB	FO	onion	SP10_2	FP	onion
Fus1	FO	onion	SP1_2	FP	onion
Fus2	FO *cepae*	onion	SP6	FP	onion
Fus3	FO	onion	K3a	FR	onion
Gr2	FO	garlic	NL41	FR	onion
Gr4	FO	garlic	NL58	FR	onion
Gr5_1	FO	garlic	NL69	FR	onion
Gr5_2	FO	garlic	A9	FS	onion
H2	FO	onion	G10	FS	onion
M9	FO	onion	C1	Fav	onion
NL34	FO	onion	LB9	Fav	leek
NL70_7	FO	onion	SP7_1	Fac	onion
NL96	FO	onion	**NRRL22544**	FO *lycopersici*	tomato
NRRL 22538	FO *cepae*	onion	**NRRL26990**	FO *freesia*	freesia
PG	FO	onion	**NRRL36311**	FO *pisi*	pea
PR5	FO	leek	**NRRL26988**	FO *freesia*	freesia
PR7	FO	leek	**Fom004**	FO *medicaginis*	alfalfa
RO2	FO	onion	**NRRL36425**	FO *lycopersici*	tomato
SH1_1	FO	shallot	**FOA4**	FO *asparagi*	asparagus
SH3	FO	shallot	**FOA5**	FO *asparagi*	asparagus
SH4	FO	shallot	**NRRL26993**	FO *gladioli*	gladiolus
SIB	FO	onion	**NRRL26222**	FO *dianthi*	carnation
SM105	FO	onion	**FO47**	FO	soil

FO: *F. oxysporum*, if f. sp. known, written in italics
FP: *F. proliferatum*, FS: *F. solani*, FR: *F. redolens*, Fav: *F. avenaceum*, Fac: *F. acuminatum*

3.3.3 Characterisation of *F. oxysporum* isolates from diseased *Allium* species and other formae speciales using housekeeping genes

Sequences for calmodulin, B-tubulin and RNA polymerase II subunit (*RPB2*) were generated in order to characterise 40 *F. oxysporum* isolates from diseased *Allium* species with reference to representatives of ff. spp. *cepae, lycopersici, freesia, pisi, medicaginis, asparagi, gladioli* and *dianthi* (Table 7). The primers used (T1/T22, CL1/CL2A, 5F2/7cR/7cF/11aR) were identical to those used in previous studies (Table 8; O'Donnell *et al*. 1998a, 2000, 2007; O'Donnell and Cigelnik, 1997). Thermal cycling conditions were as in the relevant publications. All PCR reactions, sequencing and sequence editing were prepared as described in Section 2.3.1.2.

Additionally, the identity of eleven *F. oxysporum* isolates requested from culture collections based on their *TEF* sequence similarity and ability to cause disease in the onion seedling bioassay, representing ff. spp. *lycopersici, freesia, pisi, freesia, medicaginis, lycopersici, asparagi, asparagi, gladioli, dianthi* and biocontrol agent Fo47 were confirmed based on *TEF* sequencing as in Section 2.3.1.2.

Table 8. Sequences, target gene, and origin of primers used for the characterisation of *Fusarium oxysporum* isolates from diseased onions and representatives of several formae speciales.

Primer name	Primer sequence (5`→3`)	Purpose	Target gene	Reference
T1	AACATGCGTGAGATTGTAAGT	amplification & sequencing	B-tubulin	O'Donnell & Cigelnik 1997
T22	TCTGGATGTTGTTGGGAATCC			
CL1	GAGTTCAAGGAGGCCTTCTC	amplification & sequencing	calmodulin	O'Donnell *et al*. 2000
CL2A	TTTTTGCATCATGAGTTGGAC			
5F2	GGGGWGAYCAGAAGAAGGC	amplification & sequencing	RPB2	O'Donnell *et al*. 2007
7cR	CCCATRGCTTGYTTRCCCAT			
7cF	ATGGGYAARCAAGCYATGGG			
11aR	GCRTGGATCTTRTCRTCSACC			

3.3.4 Characterisation of *F. oxysporum* isolates from diseased *Allium* species and other formae speciales using microsatellite markers

Ten previously published microsatellite markers for *F. oxysporum* were tested to determine the intraspecific variation within 40 *F. oxysporum* isolates from diseased *Allium* species and ff. spp. *cepae, lycopersici, freesia, pisi, medicaginis, asparagi, gladioli* and *dianthi* (Table 7; Bogale *et al*., 2005, 2: Almany *et al*., 2009).

DNA of 40 *F. oxysporum* isolates was extracted by using DNeasy Mini protocol (Qiagen) with minor modifications. Freeze-dried material (20 mg) was placed in a 2 ml screw-cap tube and a FastPrep instrument (MPBio) was used for trituration at 6.0 m/s for 40 seconds. Extraction was continued by proceeding to step 7 of the protocol.

Initially, ten unlabelled microsatellite primer pairs (Table 9) were tested on five *F. oxysporum* isolates representing ff. spp. *lycopersici* (FOLR1), *cepae* (Fus2 and D2) and two non-aggressive isolates from diseased onion (Fus1 and 151). Individual PCRs (10 µl) consisted of 5 µl of RedTaq (Sigma-Aldrich), 3 µl of sterile water and 0.5 µl each of the 20 µM forward/reverse primers. PCR conditions were as follows: initial denaturation for 4 minutes at 94°C, followed by 35 cycles of 30 seconds denaturation at 94°C, annealing for 30 seconds, individual annealing temperatures are in Table 9, and extension for 30 seconds at 72°C. The program ended with a 10 minutes final extension at 72°C. Polymorphisms between amplicons were evaluated based on 2% agarose gel electrophoresis (see Section 2.3.1.2). The location of primer binding sites (Table 2) was determined based on the *F. oxysporum* f. sp. *lycopersici* 4287 genome sequence by using BLAST search (Broad Institute; Altschul *et al*., 1990).

Following selection of primer pairs which were polymorphic amongst isolates, five labelled forward primer pairs (using four fluorescent labels: NED, VIC,

FAM, PET) were used to analyse 40 *F. oxysporum* isolates from diseased *Allium* species and formae speciales (Table 7). The size of labelled PCR products was determined on an ABI Prism 3100 Genetic Analyser and allele sizes assigned using GeneMarker v. 1.6 (SoftGenetics). Flexibin (Amos *et al*., 2007) was used to bin allele sizes and estimate the relative number of sequence repeats for individual *F. oxysporum* isolates at each of the five loci.

Table 9. Details of microsatellite primers used in this study.

Primer***	Sequence	Motif	Ta**	Product (bp)	Chrom osome*	Source
CH2-15 F	ATCTTCCTCACGGTTTTGGA	$(CT)_8$	59	194-213	chr 7	1
CH2-15R	TGTAGCGTAGCACAACAGTGG					1
FOL15 F	TATGGACGGATCAGGAAAGG	$(AAG)_{17}$	55	206-232	chr 1	1
FOL15 R	TCAACAACGCACTGAAGACC					1
FOL35 F-PET	GTCGTTTTCAAGGACGCACT	$(GAA)_{19}$	59	203-317	chr 1	1
FOL35 R	GGTGGCAGTTTCCTCCTTTT					1
FOL245 F-NED	TGAAAGGCGCGCTATTTAGT	$(CTT)_{14}$	55	153-258	chr 4	1
FOL245 R	GAGAGGCGGAGGAAGAAGA					1
FOL296 F	CACTGAAGGAAATGCAGCAG	$A_{23}(AAG)_{22}$	55	167-255	chr 7	1
FOL296 R	TAGGCTCTGGAGATGCTTGG					1
FOL356 F-VIC	CCTCCTGCTCTTCCTCATCTT	$(CAA)_{14}$	59	238-259	chr 2a	1
FOL356 R	CGGTATTGTTGGGGGTTTAG					1
FOL680 F-FAM	CGCAGAATGGCTCTTCAAAT	$(TTTA)_{11}$	59	200-267	chr10	1
FOL680 R	TGCAACATCATCGACCACTT					1
MB2 F-FAM	TGCTGTGTATGGATGGATGG	$(GT)_{11}(GA)_6$	55	237-275	chr 9	2
MB2 R	CATGGTCGATAGCTTGTCTCAG					2
MB13 F	GGAGGATGAGCTCGATGAAG	$(CTTGGAAGT GGTAGCGG)_{14}$	59	264-500	chr 12	2
MB13 R	CTAAGCCTGCTACACCCTCG					2
MB17 F	ACTGATTCACCGATCCTTGG	$(CA)_{21}$	55	299-339	chr 1	2
MB17 R	GCTGGCCTGACTTGTTATCG					2

*Based on *Fusarium oxysporum* f. sp. *lycopersici* genome sequence (Broad Institute) ** Ta: Annealing temperature (°C)
1: Bogale *et al*., 2005, 2: Almany *et al*., 2009 *** NED, VIC, FAM, PET: fluorescent labels

Microsatellite primers were also tested in two sets of multiplex PCRs in an attempt to make the analysis more time- and cost-effective. The M1 PCR reaction comprised FOL15, FOL 245, MB 2 and MB 17 primer pairs, while the M2 reaction contained CH2-15, FOL 35, FO 356 and FOL 680 primer pairs.

3.3.5 Development of microsatellite markers on mobile pathogenicity chromosomes to characterise *F. oxysporum* isolates from diseased *Allium* species and other formae speciales

In order to further characterise the 40 *F. oxysporum* isolates, microsatellite markers identified on a *SIX* gene encoding mobile pathogenicity chromosome (MPC) of *F. oxysporum* f. sp. *lycopersici* were tested (Ma *et al*., 2010; Broad Institute). Contigs corresponding to chromosome 14 (MPC) of *F. oxysporum* f. sp. *lycopersici* were utilized to identify microsatellite-coding sequences by using the Perfect Microsatellite Repeat Finder (Ammar, 2006). Sequences encoding short sequence repeats (SSRs) were annotated by using Blast search on NCBI database to discard those sequences encoding genes and transposable elements (NCBI; Altschul *et al*., 1990). Twelve loci were chosen for further analysis and specific primer pairs were designed by Primer3 (Rozen and Skaletsky, 2000; for primer sequences see Results).

Initially, twelve unlabelled microsatellite primer pairs (for primer sequences see Results) were tested on five *F. oxysporum* isolates representing formae speciales *lycopersici* (FOLR1), *cepae* (Fus2 and D2) and two non-aggressive isolates from diseased onion (Fus1 and 151). Individual PCRs (10 µl) consisted of 5 µl of RedTaq (Sigma-Aldrich), 3 µl of sterile water and 0.5 µl each of the 20 µM forward/reverse primers. PCR conditions were as in Section 3.3.4 with annealing temperature at 55°C. Microsatellite markers were evaluated based on 2% agarose gel electrophoresis. A single primer pair (21384F and 21384R) showed variation and resulted in a PCR product for all five *F. oxysporum* isolates. The labelled forward primer (using fluorescent label: PET) then was used to analyse 41 *F. oxysporum* isolates from diseased *Allium* species and formae speciales (Table 7). Labelled PCR products were multiplexed; Mix1: 38459, 21384 and MB2, Mix2: FOL680, FOL356, FOL35 and FOL245. The size of labelled PCR products was determined, allele sizes

and relative number of sequence repeats for individual *F. oxysporum* isolates was estimated as in 3.3.4.

3.3.6 Screening of published effector genes in *F. oxysporum* isolates collected from alliums and other host species

F. oxysporum isolates associated with diseased onion (Fus2 and A28) along with representatives of ff. spp. *lycopersici* (FOL2), *medicaginis* (Fom004) and *asparagi* (FOA5) were initially screened for the presence of a set of six effector genes; *SIX6, SIX7, FTF1, ORX1, PEP1* and *PEP2* originally used to screen *F. oxysporum* ff. spp. *lycopersici, phaseoli, cubense* and *vasinfectum*, respectively (Lievens *et al*., 2009a; Meldrum *et al*., 2012; Ramos *et al*., 2007; Chakrabarti *et al*., 2011). *F. oxysporum* f. sp. *cepae* had not been screened previously for the presence of any of these effectors. Seven published primer pairs (Table 10) were tested on this small set of isolates using the following thermal cycling conditions: initial denaturation for 1.5 minute at 94°C, followed by 35 cycles of 30 seconds denaturation at 94°C, annealing for 1.5 minutes at the 55°C and extension for 2 minutes at 68°C. The program ended with a 10 minutes final extension at 68°C. *SIX7* products successfully amplified from *F. oxysporum* isolates were sequenced. PCR reactions, sequencing and sequence editing were carried out as described in Section 2.3.1.2.

F. oxysporum isolates (175) obtained from diseased *Allium* species were screened for the presence of the *SIX7* gene using a primer pair designed by Lievens *et al*. (2009; Appendix I). Additionally 26 representatives of other ff. spp. *cepae, lycopersici* races 1 and 2, *narcissi, gladioli, asparagi, medicaginis, tulipae, dianthi, cubense, phaseoli, pisi* races 1, 2 and 5, *freesia* and biocontrol agent Fo47 were included in the *SIX7* screening.

Isolates which tested negative for the presence of *SIX7* by using the primer pair published by Lievens *et al*. (2009) were screened using newly designed *SIX7* primer pairs to confirm absence of this gene in *F. oxysporum* isolates from diseased onion (for primer sequences see Results). Primer pairs were manually designed based on alignment of *SIX7* sequences from *F. oxysporum* ff. spp. *cepae, lycopersici, lilii, narcissi, pisi* and *gladioli*. Two primer pairs were designed on the conserved regions of *SIX7*, while *F. oxysporum* f. sp. *cepae*-specific primers were designed on the variable regions. Primers were tested in combinations on nine *F. oxysporum* isolates from diseased *Allium* hosts (Fus2, D2, 151, A28, Fus1, A13, and Gr4), formae speciales *lycopersici* (FOLR1) and *medicaginis* (Fom004). PCR reactions were set up as described in Section 2.3.1.2.

Table 10. Sequences and references of seven effector gene-specific primer pairs used for this study.

Primer	5' → 3' Sequence	Target	References
SIX6-F1	CTCTCCTGAACCATCAACTT	Secreted in xylem protein 6	Lievens *et al*., 2009a
SIX6-R1	CAAGACCAGGTGTAGGCATT		
SIX7-F1	CATCTTTTCGCCGACTTGGT	Secreted in xylem protein 7	Lievens *et al*., 2009a
SIX7-R1	CTTAGCACCCTTGAGTAACT		
SIX8-F1	TCGCCTGCATAACAGGTGCCG	Secreted in xylem protein 8	Meldrum *et al.*, 2012
SIX8-R1	TTGTGTAGAAACTGGACAGTCGATGC		
FTF1-F1	CGTTTCCCTACTAGATCACACCCA	*Fusarium* transcription factor 1	Ramos *et al*., 2007
FTF1-R1	GCTCCTGTATCTCCTCGGAG		
ORX1-F1	CCAGGCCATCAAGTTACTC	Putative oxidoreductase 1	Chakrabarti *et al.*, 2011
ORX1-R1	TCTCCAATATGGCAGATTGTG		
PEP1-F	TCATGCTCTACCCACGCTTT	Putative effector protein 1	Chakrabarti *et al*., 2011
PEP1-R	CGGACTGCAAGTGCCTTATC		
PEP2-F	ACTCAACATGAAGGTTACGG	Putative effector protein 2	Chakrabarti *et al*., 2011
PEP2-R	CTCCGCGTAGTAATAGTTTCTC		

F or f: forward primer, R or r: reverse primer

PCR cycling conditions were as follows: initial denaturation for 5 minutes at 95°C, followed by 40 cycles of 1 minute denaturation at 93°C, annealing for 1 minute at the 56°C and extension for 1 minute at 72°C. The program ended with a 10 minutes final extension at 72°C.

3.3.7 Cloning of the homologous *SIX7* sequence of *F. oxysporum* f. sp. *cepae*

To confirm that the homologous *SIX7* sequence of *F. oxysporum* f. sp. *cepae* was not an artefact of incorrect sequencing, the *SIX7* genes of *F. oxysporum* f. sp. *cepae* (Fus2 isolate) and *F. oxysporum* f. sp. *lycopersici* race 2 (FOL2 isolate) were cloned and sequenced.

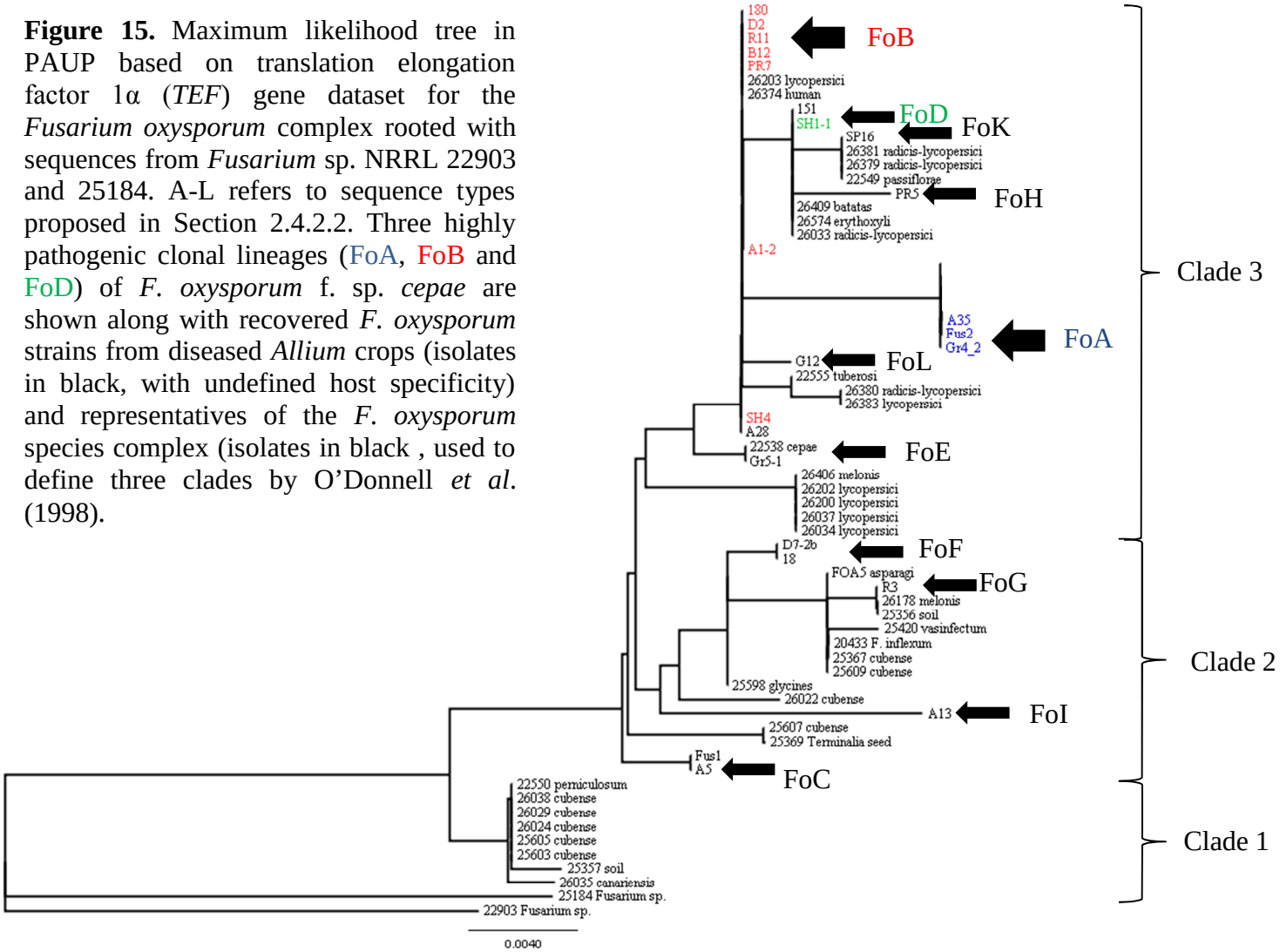
PCR products of Fus2 and FOL2 isolates obtained using SIX7-F1 and SIX7R1 primers (Lievens *et al*., 2009a; Table 7) were cleaned using a Qiagen PCR purification kit following the manufacturer's recommendations. A TOPO® TA cloning kit (Invitrogen) was used to ligate PCR products into pCRII TOPO® vectors. Ligations were carried out at 14°C for 4 hours. Chemical transformation was carried out using TOP10F' chemically competent cells (Invitrogen) following the producers protocol. 20 µl suspension was used each for the inoculation of LB plates containing ampicillin (100 µg/ml) to select *SIX7*-amplicon-containing white *E. coli* cells (Sambrook *et al*., 1989). Ten white colonies were sub-cultured each into 2ml tubes containing LB medium amended with ampicillin (100 µg/ml) and grown for 24 hours at 37°C. Plasmid DNA was then isolated using a QIAprep Spin Miniprep Kit (Qiagen). Positive transformants were identified by PCR using M13 forward and reverse primers targeting pCRII plasmid sequence following Invitrogen's recommendations. Amplicons were purified, sequenced and analysed as in 2.3.1.2.

3.4 RESULTS

3.4.1 Cladistic analysis of *F. oxysporum* isolates associated with diseased *Allium* species and different formae speciales based on *TEF* sequences

A maximum likelihood tree was constructed in PAUP using *TEF* to study the genetic organisation of FOC within the *F. oxysporum* species complex (Figure 15). Sequences (28) of *F. oxysporum* isolated from diseased *Allium* crops representing eleven *TEF* sequence types (FoA-FoL, see Chapter 2) were used in conjunction with the *TEF* dataset generated by O'Donnell *et al*. (1998a; Figure 1) to study the genealogies of isolates associated with diseased *Allium* species. Sequences fell into three clades of the *F. oxysporum* species complex and the topology of the maximum likelihood tree agreed with a parsimonious tree published by O'Donnell *et al*. (1998a, Figure 1). Representatives of *TEF* sequence types FoA, FoB, FoD, FoE, FoH, FoK, FoL (Section 2.4.2.2) from diseased onion fell into Clade 3, while isolates belonging to *TEF* sequence types FoC, FoF, FoG and FoI fell into Clade 2. *F. oxysporum* isolates belonging to ST FoB shared the same *TEF* sequence with a *F. oxysporum* f. sp. *lycopersici* and a human pathogen isolate. Similarly, FoD (*batatas, erythoxyli, radicis-lycopersici*), FoK (*radicis-lycopersici* and *passiflorae*) and FoG (*melonis* and soil isolate) were found polyphyletic.

Figure 15. Maximum likelihood tree in PAUP based on translation elongation factor 1α (*TEF*) gene dataset for the *Fusarium oxysporum* complex rooted with sequences from *Fusarium* sp. NRRL 22903 and 25184. A-L refers to sequence types proposed in Section 2.4.2.2. Three highly pathogenic clonal lineages (FoA, FoB and FoD) of *F. oxysporum* f. sp. *cepae* are shown along with recovered *F. oxysporum* strains from diseased *Allium* crops (isolates in black, with undefined host specificity) and representatives of the *F. oxysporum* species complex (isolates in black , used to define three clades by O'Donnell *et al.* (1998).



Sequences of 32 *F. oxysporum* isolates associated with *Allium* species representing TEF STs FoA – FoM) were compared with the *TEF* sequences of 100 isolates (from GenBank) representing 39 different formae speciales using neighbour-joining method (Figure 16). The *TEF* sequences formed three clades and the distribution of *TEF* sequence types agreed with the maximum likelihood tree (Figure 15). The diversity of isolates from *Allium* species indicates the polyphyletic origin of FOC. Moreover, more than one formae speciales occurred in each clonal lineage (Figure 16). For example, clonal lineage FoA comprises identical *TEF* sequences of cultures pathogenic to *Allium* species, tomato (*Solanum lycopersicum*), peas (*Pisum sativum*), freesia (*Freesia* sp.), alfalfa (*Medicago sativa*) and asparagus (*Asparagus officinalis*). The same trend can be seen in clonal lineage FoB, representatives of formae speciales *cepae, lycopersici, batatas* along with a human pathogenic strain share identical *TEF* sequences. Further examples are in clonal lineage FoD which consists of representatives of ff. spp. *cepae, niveum, bouvardiae* and *chrysanthemi*, FoE (*chrysanthemi, tulipae, vasinfectum, dianthi* and *gladioli*), FoI (*pisi*), FoF (*lilii*), FoC (*gladioli*) and FoG (*melonis, lini, dianthi* and *cucumerinum*).

The results suggested that *F. oxysporum* f. sp. *cepae* (FOC) and isolates belonging to other formae speciales can have identical *TEF* sequence (Figure 15 and Figure 16). Five *F. oxysporum* isolates were requested from culture collections based on similarity to *TEF* sequence type FoA (NRRL22544 - *lycopersici*, NRRL26990 – *freesia*, NRRL36311 – *pisi*, FOA4 – *asparagi* and Fom004 – *medicaginis*). Five additional *F. oxysporum* isolates were requested based on symptoms caused by them (NRRL26993 – *gladioli*, NRRL26988 – *freesia*, NRRL36425 – *lycopersici*, FOA5 – *asparagi* and NRRL26222 – *dianthi*).

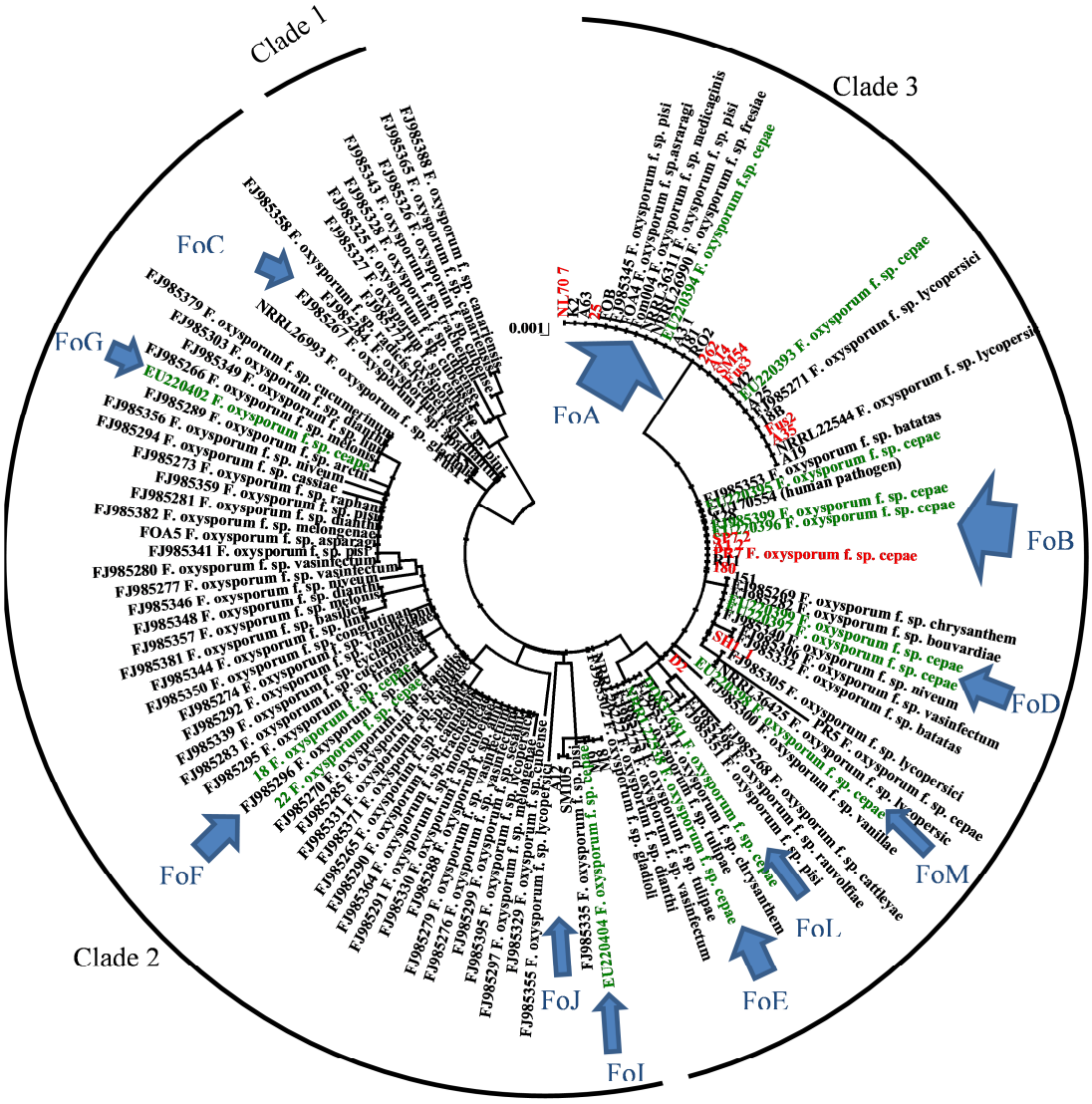


Figure 16. Cladogram of representatives of *Fusarium oxysporum* formae speciales based on *TEF*. Consensus tree is shown with bootstrap values from 1000 replications. GenBank accession number is indicated for each reference sequence. Isolates from diseased *Allium* species are shown in red. Sequences of *Fusarium oxysporum* f. sp. *cepae* isolates published by various groups are in green. FoA - FoM refer to sequence types (STs) proposed in chapter 2 which can also be seen as clonal lineages. Moreover, the three clades of the *F. oxysporum* species complex proposed by O'Donnell *et al*. (1998a) are shown.

SUMMARY

TEF sequence of *F. oxysporum* isolates from diseased *Allium* crops were analysed by neighbour-joining and maximum likelihood methods and isolates formed two main clades, Clade 2 and Clade 3 having been determined by O'Donnell *et al*. (1998a). The diversity of isolates from *Allium* species indicated the polyphyletic origin of FOC. Moreover, more than one formae speciales occurred in each clonal lineage; FOC and isolates belonging to other formae speciales can share identical *TEF* sequence. For example, the most commonly occurring ST FoA (78%) comprises identical *TEF* sequences of cultures pathogenic to *Allium* species, tomato, freesia, peas and alfalfa.

To clarify that these findings were robust, various molecular markers such as three housekeeping genes (RPB2, calmodulin and β-tubulin), six microsatellite markers and effector gene (*SIX7*) as well as pathogenicity on onion seedlings (see next Section) were tested on 40 *F. oxysporum* isolates recovered from diseased *Allium* species and representing several sequence types. Additionally, representatives of other formae speciales grouped together with clonal lineages of FOC were included in these experiments.

3.4.2 Onion seedling assay using *Fusarium* isolates from diseased *Allium* species and different formae speciales

Disease symptoms appeared as pre- and post-emergence damping-off four weeks after inoculation and as the disease progressed onion leaves became wilted and yellow (Figure 17). In some cases, white/pink mycelium was observed on the surface of the necrotic onion leaves. Untreated plants remained symptomless. The survival index (angular transformed percentage survival) was calculated of each treatment. The REML analysis showed that there was a significant difference ($P \leq$

0.001) between treatments (Table 11, Appendix IV). The final survival index was 12.1 for the very aggressive Fus2 isolate (positive standard), 38.3 for the biocontrol agent Fo47 strain (negative standard) and 57.5 for the untreated. These treatments were significantly different from each other as the least significant difference was 11.4. Continuous variation was found among the 74 *Fusarium* isolates tested (Figure 18).

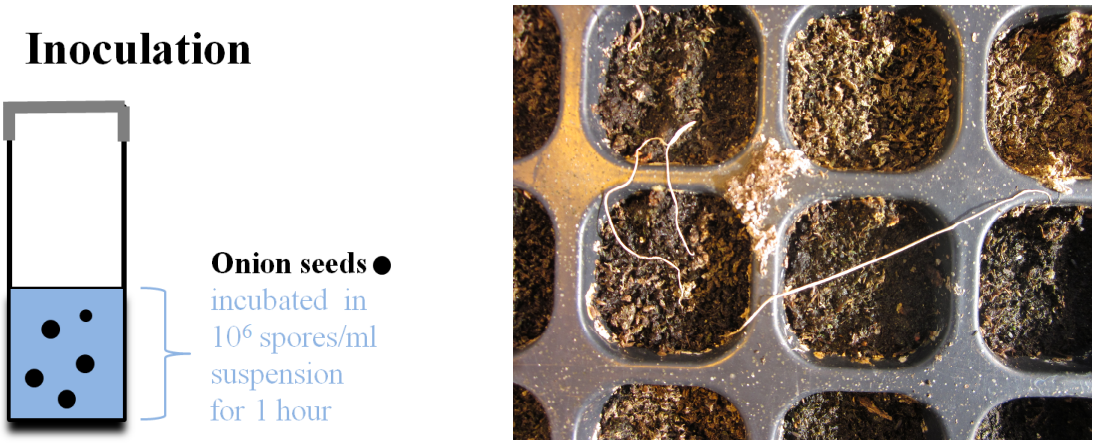


Figure 17. Inoculation method and damping-off symptoms of onion seedlings four weeks after sowing inoculated onion seeds.

Isolates with survival index not significantly different (overall maximum l.s.d 5%: 11.4) from the isolate Fus2 according to the REML analysis were considered very aggressive. Isolates with survival index significantly different from Fo47 treatment according to the REML analysis were considered as aggressive. Isolates with survival index not significantly different from Fo47 treatment and untreated controls were considered non-aggressive.

F. oxysporum isolates from diseased *Allium* species

Based on the seedling assay, 20 isolates out of 42 *F. oxysporum* isolates from diseased *Allium* species were very aggressive, three moderately aggressive while the remaining 19 isolates were found non-aggressive (Table 11, Figure 18). The very aggressive *F. oxysporum* isolates all originated from onion except for one from leek (PR7) and one from shallot (SH1-1) and originated from the UK, the Netherlands, Spain, USA and Chile, respectively. Non-aggressive strains were from onion, leek, shallot, garlic and Welsh onion grown in the UK, the Netherlands, Italy, Japan, Spain, Germany and Australia. However, three *F. oxysporum* isolates (NRRL 22538, 22, 18) defined as non-aggressive based on the seedling assay were originally identified as FOC in previous studies (Dissanayake *et al*, 2009ab, Agricultural Research Service, ARS).

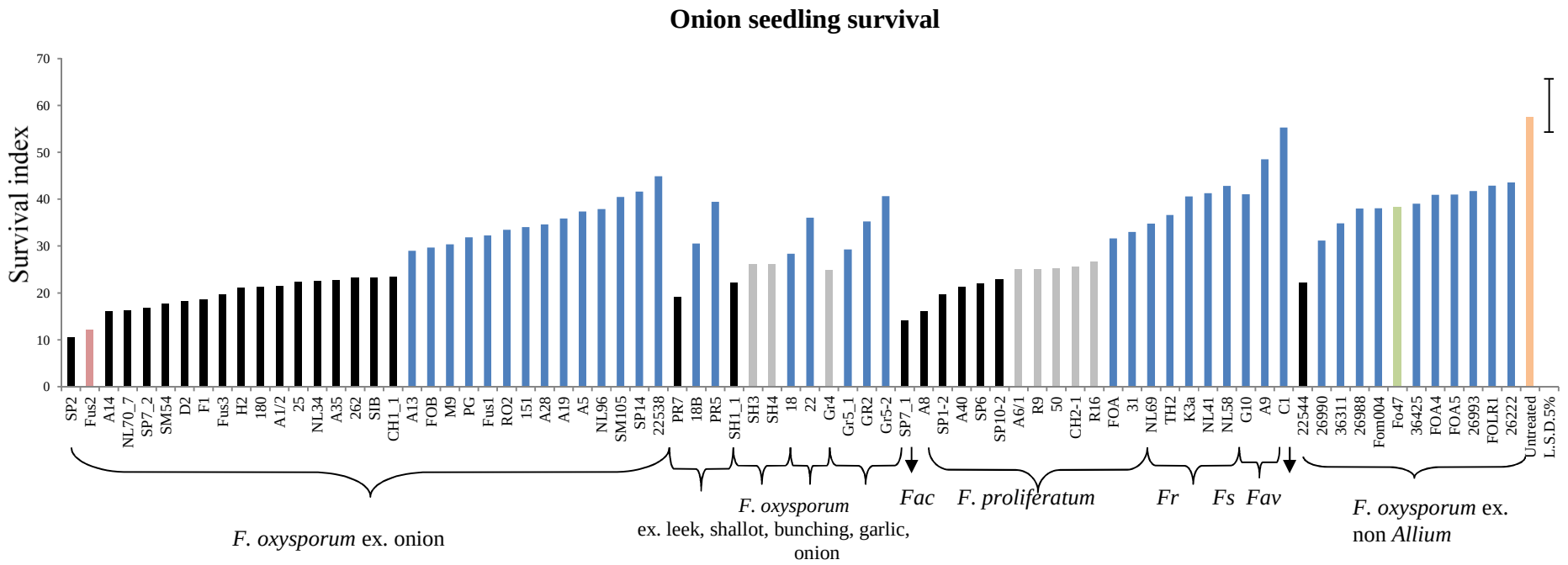


Figure 18. Survival index (angular transformed percentage onion seedling survival) of 74 *Fusarium* isolates representing isolates from diseased *Allium* species and eight formae speciales.
Means adjusted in REML, general linear model. L.S.D.s calculated at 5% F pr.<0.001
Fac: F. acuminatum; Fr: F. redolens, Fs: F. solani; Fav: F. avenaceum
Black isolates were not significantly different (**maximum overall l.s.d 5%: 11.4**) from **Fus2 treatment** according to REML analysis. **Grey isolates** were significantly different (l.s.d 5%) from **Fo47 treatment** according to REML analysis. **Blue isolates** were not significantly different (l.s.d 5%) from **Fo47 treatment** and not significantly different (l.s.d 5%) from **Untreated** according to REML analysis

Table 11. Seventy-four *Fusarium* isolates used in onion seedling assay with reference to host, TEF sequence type and survival index (Si) of seedlings, respectively.

Isolate	Host	TEF	Si*	Isolate	Host	TEF	Si*
SP2	**onion**	**FoA**	**10.4**	**NL96**	**onion**	**FoA**	**37.9**
Fus2	**onion**	**FoA**	**12.1**	**PR5**	**leek**	**FoH**	**39.5**
A14	**onion**	**FoA**	**16.0**	**SM105**	**onion**	**FoI**	**40.5**
NL70_7	**onion**	**FoA**	**16.2**	**SP14**	**onion**	**FoB**	**41.6**
SP7_2	**onion**	**FoB**	**16.8**	**22538**	**onion**	**FoE**	**44.9**
SM54	**onion**	**FoA**	**17.7**	**A8**	**onion**	**FP2**	**16.1**
D2	**onion**	**FoB**	**18.2**	**SP1_2**	**onion**	**FP3**	**19.6**
F1	**onion**	**FoA**	**18.6**	**A40**	**onion**	**FP1**	**21.3**
PR7	**leek**	**FoB**	**19.1**	**SP6**	**onion**	**FP3**	**22.0**
Fus3	**onion**	**FoA**	**19.7**	**SP10_2**	**onion**	**FP3**	**22.8**
H2	**onion**	**FoA**	**21.0**	**A6/1**	**onion**	**FP2**	**25.0**
180	**onion**	**FoB**	**21.2**	**R9**	**onion**	**FP1**	**25.1**
A1/2	**onion**	**FoB**	**21.4**	**50**	**onion**	**FP1**	**25.2**
SH1_1	**shallot**	**FoD**	**22.2**	**CH2-1**	**onion**	**FP1**	**25.5**
25	**onion**	**FoA**	**22.4**	**R16**	**onion**	**FP1**	**26.7**
NL34	**onion**	**FoA**	**22.6**	**FOA**	**onion**	**FP1**	**31.6**
A35	**onion**	**FoA**	**22.7**	**31**	**onion**	**FP1**	**33.0**
262	**onion**	**FoA**	**23.3**	**SP7_1**	**onion**	**Fac**	**14.1**
SIB	**onion**	**FoA**	**23.3**	**G10**	**onion**	**FS2**	**41.1**
CH1_1	**onion**	**FoA**	**23.4**	**A9**	**onion**	**FS8**	**48.5**
Gr4	**garlic**	**FoA**	**24.9**	**NL69**	**onion**	**FR1**	**34.8**
SH3	**shallot**	**FoD**	**26.1**	**TH2**	**onion**	**FR1**	**36.6**
SH4	**shallot**	**FoB**	**26.2**	**K3a**	**onion**	**FR2**	**40.6**
18	**Welsh onion**	**FoF**	**28.4**	**NL41**	**onion**	**FR1**	**41.3**
A13	**onion**	**FoI**	**29.0**	**NL58**	**onion**	**FR1**	**42.8**
Gr5_1	**garlic**	**FoE**	**29.3**	**C1**	**onion**	**FA2**	**55.3**
FOB	**onion**	**FoA**	**29.7**	**NRRL22544**	**tomato**	**A**	**22.2**
M9	**onion**	**FoI**	**30.4**	**NRRL26990**	**freesia**	**A**	**31.2**
18B	**onion**	**FoA**	**30.5**	**Fo47**	**biocontrol**	**N**	**38.3**
PG	**onion**	**FoD**	**31.8**	**NRRL36311**	**pea**	**A**	**34.9**
Fus1	**onion**	**FoC**	**32.3**	**FOA4**	**asparagus**	**A**	**41.0**
151	**onion**	**FoD**	**34.0**	**Fom004**	**alfalfa**	**A**	**38.0**
A28	**onion**	**FoB**	**34.6**	**NRRL26993**	**gladiolus**	**C**	**41.7**
Gr2	**garlic**	**FoB**	**35.2**	**NRRL26988**	**freesia**	**L**	**38.0**
A19	**onion**	**FoA**	**35.9**	**NRRL36425**	**tomato**	**M**	**39.0**
22	**Welsh onion**	**FoF**	**36.1**	**FOA5**	**asparagus**	**N**	**41.0**
A5	**onion**	**FoC**	**37.4**	**NRRL26222**	**carnation**	**O**	**43.6**

FP: *F. proliferatum*, FA: *F. acuminatum*, FS: *F. solani*, FR: *F. redolens*, FA: *F. avenaceum*
Si: survival index, percentage onion seedling survival (angular transformed)
Black isolates were not significantly different (maximum overall l.s.d 5%: 11.4) from **Fus2 treatment** according to REML analysis
Grey isolates were significantly different (l.s.d 5%) from **Fo47 treatment** according to REML analysis
Blue isolates were not significantly different (l.s.d 5%) from **Fo47 treatment** and not significantly different (l.s.d 5%) from **Untreated** according to REML analysis (Di of Untreated was 57.54)
***overall maximum l.s.d. 5%**: approximate least significant differences (5% level) of REML means
TEF: translation elongation factor (*TEF*) sequence types defined in Chapter 2, (see Appendix II-III)

F. oxysporum isolates representing different formae speciales

Eleven *F. oxysporum* isolates representing seven formae speciales *lycopersici, freesia, pisi, asparagi, medicaginis, gladioli* and *dianthi* and biocontrol strain Fo47 (negative standard) were tested on onion seedlings (Table 11, Figure 18). Nine out of ten treatments (isolates) were also not significantly different from the negative standard (Fo47) treatment. Interestingly, isolate NRRL 22544 was not significantly different from the positive standard (Fus2) treatment although it was originally identified as f. sp. *lycopersici*.

Other *Fusarium* species

Five *F. proliferatum* isolates from the UK and Spain (A8, A40, SP1-2, SP6 and SP10-2) were found very aggressive and four from British, Spanish and American onions were moderately aggressive. Two *F. proliferatum* (FOA and 31), two *F. solani* (G10 and A9), five *F. redolens* (NL69, TH2, K3a, NL41 and NL58) and one *F. avenaceum* (C1) isolate tested were non-aggressive in onion seedlings. One *F. acuminatum* isolate (SP7-1) was very aggressive.

Additionally, 14 *F. oxysporum* isolates from diseased *Allium* species and formae speciales *asparagi, lycopersici, medicaginis, pisi, freesia* and a biocontrol strain (Fus2, 180, D2, NL96, A13, Fus1, A5, FOA5, Fom004, NRRL 36425, NRRL 26988, NRRL 26222, NRRL 36311 and Fo47) were tested using the same protocol as before, except the final concentrations were fifty times less (2×10^4 spores/ml) than in the inoculum dose in the original protocol (10^6 spores/ml, Appendix IV). All isolates were less aggressive at this lower dose. No significant difference was observed between untreated (water immersed) and treatments with twelve isolates (NL96, A13, Fus1, A5, FOA5, Fom004, NRRL 36425, NRRL 26988, NRRL 26222,

NRRL 36311 and Fo47), which were therefore considered to be non–pathogenic in seedlings of standard susceptible cultivars of onion. In the same experiment, two isolates (D2 and 180) isolates were as aggressive in onion seedlings as the standard pathogenic isolate Fus2.

Combining the the *TEF* sequence data with the results of the pathogenicity assays showed most clades or sequence types did not correlate with pathogenic ability on onion (Table 11). There are three exceptions: sequence types FoC, FoI and FoJ were found non-aggressive in onion seedlings (although only one or two isolates were chosen from each of these STs).

SUMMARY

In total, 63 *Fusarium* isolates associated with basal rot of *Allium* species and 11 isolates associated with other hosts were tested in a rapid seedling assay to define their aggressiveness in onion seedlings. *F. oxysporum* isolates associated with onion basal rot (42) belong to at least nine different *TEF* sequence types (STs FoA-FoL). However, *F. oxysporum* isolates from *Allium* species that tested strongly or moderately aggressive in onion seedlings (termed as FOC) were found only in three STs (FoA, FoB and FoD). Five *F. proliferatum* and one *F. acuminatum* isolate tested were very aggressive. Additionally, four *F. proliferatum* isolates were moderately aggressive, while two *F. proliferatum*, two *F. solani*, five *F. redolens* and one *F. avenaceum* isolate tested were non-aggressive in onion seedlings. Interestingly, a *F. oxysporum* isolate (NRRL 22544) was not significantly different from the positive standard (Fus2) treatment although it was originally identified as f. sp. *lycopersici*.

3.4.3 Molecular characterisation of *F. oxysporum* isolates using housekeeping genes

Three housekeeping genes (calmodulin, B-tubulin and *RPB2*) were used for the characterisation of 40 *F. oxysporum* strains representing formae speciales *cepae, lycopersici, pisi, medicaginis, asparagi, freesia, dianthi, gladioli* along with non-aggressive isolates recovered from diseased *Allium* species and biocontrol strain Fo47 (Table 12). Additionally *TEF* sequences of these isolates were also included in this analysis as it is a housekeeping gene. Isolates were divided into sequence types (STs) based on the individual and combined datasets. *TEF* (14 STs) and *RPB2* (13 STs) were more informative compared to calmodulin (7 STs) and β-tubulin (7 STs) (Table 12). The combination of the four datasets led to the identification of 19 STs among the 40 *Fusarium oxysporum* isolates analysed (Table 12).

The combined dataset was analysed in relation to host and the results of the onion seedling assay (Figure19A). Several sequence types were exclusively found from onion (ST6, ST7, ST9, ST10, ST13 and ST19), carnation (ST18), freesia (ST15), tomato (ST16), asparagus (ST17), garlic (ST8), bunching onion (ST11), leek (ST12) and the biocontrol strain (ST14). On the other hand, a few sequence types were found among various formae speciales e.g. ST1 comprised formae speciales *cepae*, *lycopersici* and *asparagi* isolates; ST2 formae speciales *cepae, medicaginis* and *pisi*; ST3 included isolates from onion, leek and freesia and ST5 consisted of formae speciales *cepae* and *gladioli* isolates.

Table 12. Multilocus sequence type for 40 *Fusarium oxysporum* isolates from various host plants with reference to survival index, based on *TEF*, *RPB2*, tubulin (*TUB*), calmodulin (*CAL*) and combined sequence types (STs).

Culture	Host	Survival index*	TEF	RPB2	CAL	TUB	Combined
Fus2	onion	12.1	A	A	A	A	ST1
Fus3	onion	**19.7**	A	A	A	A	ST1
NRRL22544	tomato (or onion**)	**22.2**	A	A	A	A	ST1
25	onion	**22.4**	A	A	A	A	ST1
A35	onion	**22.7**	A	A	A	A	ST1
262	onion	**23.3**	A	A	A	A	ST1
FOB	onion	29.7	A	A	A	A	ST1
FOA4	asparagus	41.0	A	A	A	A	ST1
A14	onion	**16.0**	A	E	A	A	ST2
N70_7	onion	**16.2**	A	E	A	A	ST2
SIB	onion	**23.3**	A	E	A	A	ST2
NRRL36311	pea	34.9	A	E	A	A	ST2
A19	onion	35.9	A	E	A	A	ST2
Fom004	alfalfa	38.1	A	E	A	A	ST2
SM54	onion	**17.7**	A	I	A	A	ST3
18B	leek	30.5	A	I	A	A	ST3
NRRL26990	freesia	31.2	A	I	A	A	ST3
D2	onion	**18.2**	B	A	A	A	ST4
PR7	leek	**19.1**	B	A	A	A	ST4
180	onion	**21.2**	B	A	A	A	ST4
A1/2	onion	**21.4**	B	A	A	A	ST4
Fus1	onion	32.3	C	H	D	D	ST5
A5	onion	37.4	C	H	D	D	ST5
NRRL26993	gladiolus	41.7	C	H	D	D	ST5
SH1_1	shallot	**22.2**	D	F	B	A	ST6
151	onion	34.0	D	F	B	A	ST6
A13	onion	29.0	I	K	E	C	ST7
SM105	onion	40.5	I	K	E	C	ST7
Gr4	garlic	24.9	A	C	A	A	ST8
A28	onion	34.6	B	C	A	A	ST9
PG	onion	31.8	D	B	A	A	ST10
22	Welsh onion	36.1	F	M	F	B	ST11
PR5	leek	39.5	H	B	A	A	ST12
M9	onion	30.4	J	K	E	C	ST13
FO47	biocontrol	38.3	K	D	A	A	ST14
NRRL26988	freesia	38.0	L	J	D	E	ST15
NRRL36425	tomato	39.1	M	E	A	A	ST16
FOA5	asparagus	41.0	N	L	G	G	ST17
NRRL26222	carnation	43.6	O	J	D	F	ST18
NRRL22538	onion	44.9	P	G	C	A	ST19
No. of sequence types			14	13	7	7	19

TEF: translation elongation factor, CAL: calmodulin, TUB: β-tubulin, RPB2: RNA polymerase II 2nd largest subunit

Survival index: percentage onion seedling survival (angular transformed data)

Black isolates were not significantly different (maximum overall l.s.d 5%: 11.393) from **Fus2 treatment** according to REML analysis; **Grey isolates** were significantly different (l.s.d 5%) from **Fo47 treatment** according to REML analysis; **Blue isolates** were not significantly different (l.s.d 5%) from **Fo47 treatment** and not significantly different (l.s.d 5%) from **Untreated** according to REML analysis (Di of Untreated was 57.54)

***overall maximum l.s.d. 5%**: approximate least significant differences (5% level) of REML means

** Originally designated as tomato pathogen but was very aggressive in onion seedlings

The combined sequence dataset was analysed in relation to the results of the onion seedling assay (Figure 19, Table 12). For interpretation of results, isolates tested in the pathogenicity assay were divided into two groups: “aggressive” and “non-aggressive” based on statistical similarity to the Fo47 treatment. Although it has to be noted that it is a very arbitrary division considering that *Fusarium* isolates showed continuous variation in terms of aggressiveness in onion seedlings. Thirteen sequence types were exclusively found to be non-aggressive (ST5, ST7, ST9- to ST19), while only two were considered as aggressive in onion seedlings (ST4 and ST8). Four sequence types comprised both aggressive and non-aggressive isolates (ST1, ST2, ST3 and ST6). ST1 comprised isolates from tomato, asparagus and onion and surprisingly the isolate from tomato was found to be very aggressive, while one of the onion isolates and the isolate from asparagus were found non-aggressive (Table 12). ST2 consisted of non-aggressive pea, alfalfa and one onion isolates, whereas the very aggressive isolates were all recovered from onion. ST3 contained isolates from onion, leek and freesia. The leek and freesia isolates were non-aggressive unlike the onion isolate. The shallot isolate was found to be aggressive and the onion isolate was non-aggressive in ST6 (Table 12).

In summary, calmodulin, B-tubulin and *RPB2* are the three additional housekeeping genes used in combination for the characterisation of 40 *F. oxysporum* strains. This led to the identification of 19 sequence types (STs), but only two of them (ST4 and ST8) correlated with aggressiveness in onion seedlings. Overall, *TEF, RPB2,* B-tubulin and calmodulin were not useful for the unambiguous identification of formae speciales.

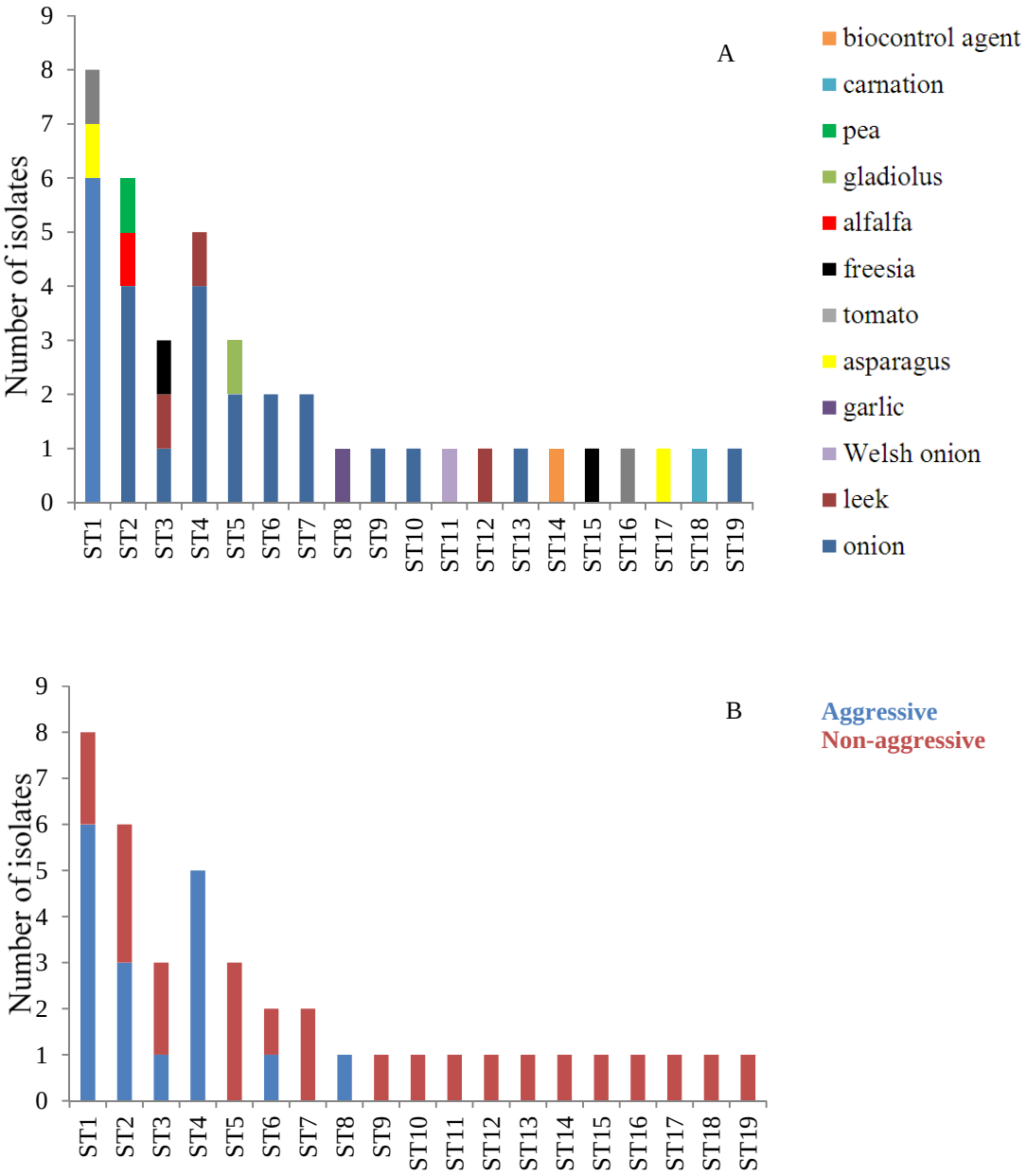


Figure 19. Frequency of sequence types (STs) based on *TEF*, *RPB2*, calmodulin and β-tubulin sequences of 40 *Fusarium oxysporum* isolates from diseased *Allium* and other formae speciales for **A** different host plants and **B** relation to onion seedlings. ST1–ST19: sequence types based on combined dataset of *TEF*, *RPB2*, calmodulin and β-tubulin sequences (see Table 12).

3.4.4 Molecular identification of *F. oxysporum* isolates using published microsatellite markers

Ten previously published (Bogale *et al*., 2005, Almany *et al.*, 2009) microsatellite markers were tested to characterise *F. oxysporum* isolates associated with diseased *Allium* species and different formae speciales.

Ten unlabelled primer pairs amplifying microsatellites were tested on five *F. oxysporum* isolates, representing formae speciales *cepae, lycopersici* and non-aggressive isolates from onion, initially to define the optimal annealing temperatures. The MB 9 primer pair did not give any amplicons and a BLAST search of the MB 9 primer sequences did not give any hits against the *F. oxysporum* genome sequence either. MB13 did not amplify from all isolates. Gel electrophoresis of products suggested that FOL 15, FOL 245, FOL 296, FOL 35 were more informative than FOL296, FOL 680, CH2-15, FOL 356, MB 2, MB 14 and MB 17 (data not shown).

The FOL 245 primer pair did not give a band and MB17 produced a faint band in M1 multiplex PCR and the detection of products of M2 multiplex PCR based on electrophoresis was not informative, therefore primer pairs were used in individual reactions (Figure 20), although, FOL15 and MB2 could be used in a multiplex PCR format.

MB2 (FAM), FOL680 (FAM), FOL356 (VIC), FOL35 (PET) and FOL245 (NED) were chosen for further analysis on the basis of their chromosomal location e.g. each of these markers targeting differing chromosomes.

The 40 *F. oxysporum* isolates (Table 7) from diseased *Allium* species and several different formae speciales were analysed by five microsatellites markers. The fragment lengths varied between MB2: 241-274, FOL680: 193-204, FOL356: 120-326, FOL35: 212-255 and FOL245: 135-267 (Appendix V). Allele size variation of

FOL356 and FOL245 is more likely to be the result of insertion rather than variation in number of microsatellite repeats.

FOL 356 (16 haplotypes), FOL 245(12 haplotypes), MB2 (10 haplotypes) and FOL35 (9 haplotypes) were more informative than, FOL680 (3 haplotypes). In total 26 unique haplotypes were found among 40 *F. oxysporum* isolates from diseased *Allium* species and different formae speciales (Table 13).

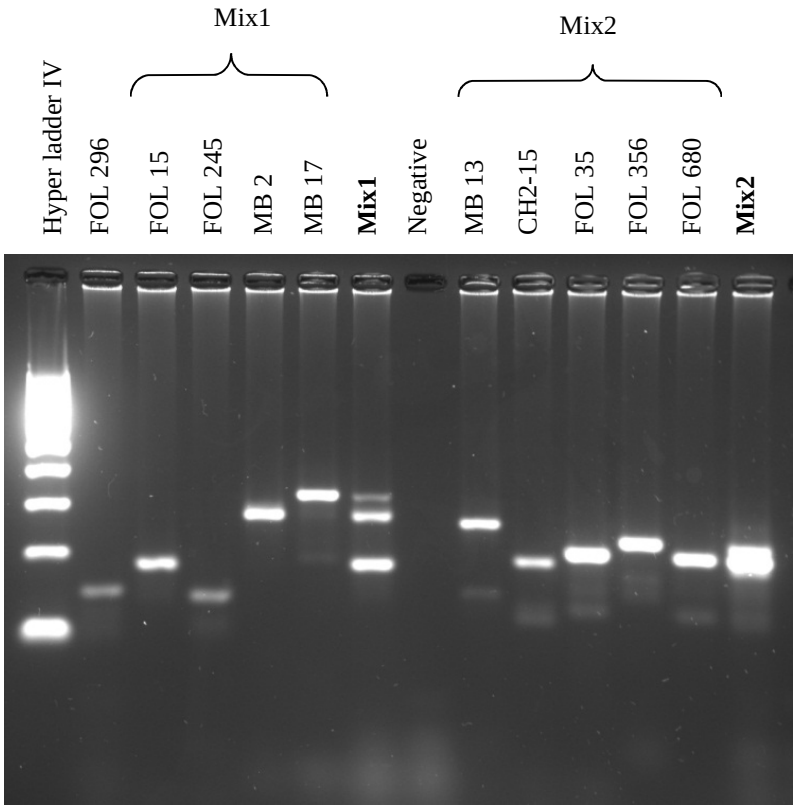


Figure 20. Gel electrophoresis photo of two multiplex microsatellite PCRs in comparison to individual PCRs. Microsatellite primers individually (FOL 15, FOL 245, FOL 296, FOL 35, FOL296, FOL 680, CH2-15, FOL 356, MB 2, MB 14 and MB 17) and in multiplex format (M1 and M2) using Fus2 isolate as template.

Table 13. Microsatellite haplotypes for *Fusarium oxysporum* isolates affecting various host plants with reference to survival index (SI), estimated repeat numbers of five individual microsatellites markers and combined haplotypes (HTs).

Culture	Host	SI	Microsatellite markers (repeat numbers*)					
			FOL680	MB2	FOL35	FOL245	FOL356	HTs
NRRL26988	freesia	**38.0**	2	1	1	29	28	HT1
NRRL26222	carnation	**43.6**	2	1	1	39	28	HT2
NRRL22538	onion	**44.9**	2	2	8	3	30	HT3
M9	onion	**30.4**	1	4	3	43	28	HT4
SM105	onion	**40.5**	1	4	3	31	28	HT5
A13	onion	**29.0**	1	4	3	32	28	HT6
22	Welsh onion	**36.0**	1	6	2	25	62	HT7
PR5	leek	**39.5**	2	5	9	1	63	HT8
PG	onion	**31.8**	3	6	4	1	49	HT9
A14	onion	**16.0**	3	6	4	1	43	HT10
SIB	onion	**23.3**	3	6	4	1	43	HT10
A19	onion	**35.9**	3	6	4	1	43	HT10
Fom004	alfalfa	**38.0**	3	6	5	1	43	HT11
FO47	biocontrol agent	**38.3**	2	8	15	26	1	HT12
Fus2	onion	**12.1**	2	10	7	1	41	HT13
Fus3	onion	**19.7**	2	10	7	1	41	HT13
NRRL22544	tomato	**22.2**	2	10	7	1	41	HT13
25	onion	**22.4**	2	10	7	1	41	HT13
A35	onion	**22.7**	2	10	7	1	41	HT13
262	onion	**23.3**	2	10	7	1	41	HT13
Gr4	garlic	**24.9**	2	10	7	1	41	HT13
FOB	onion	**29.7**	2	10	7	1	41	HT13
SM54	onion	**17.7**	2	10	7	27	49	HT14
A28	onion	**34.6**	2	10	7	1	60	HT15
FOA4	asparagus	**41.0**	2	10	7	31	66	HT16
NRRL26990	freesia	**31.2**	2	10	7	27	69	HT17
SH1_1	shallot	**22.2**	2	10	4	1	33	HT18
151	onion	**34.0**	2	10	4	1	33	HT18
PR7	leek	**19.1**	3	10	5	28	35	HT19
A1/2	onion	**21.4**	3	10	5	28	35	HT19
D2	onion	**18.2**	3	10	7	28	35	HT20
180	onion	**21.2**	3	10	7	28	35	HT20
N70_7	onion	**16.2**	3	10	4	1	43	HT21
18B	leek	**30.5**	3	10	7	1	40	HT22
NRRL36425	tomato	**39.0**	3	10	7	1	55	HT23
NRRL36311	pea	**34.9**	3	11	7	1	60	HT24
FOA5	asparagus	**41.0**	1	13	3	34	31	HT25
Fus1	onion	**32.3**	2	17	4	1	28	HT26
A5	onion	**37.4**	2	17	4	1	28	HT26
NRRL26993	gladiolus	**41.7**	2	17	4	1	28	HT26
No. of haplotypes			3	10	9	12	16	26

Microsatellites used: MB2, FOL680, FOL356, FOL35 and FOL245 (Bogale *et al*., 2005, Almany *et al.*, 2009)
* Repeat number variation of FOL356 and FOL245 is more likely to be the result of insertion rather than variation in number of microsatellite repeats.
SI: Survival index: percentage onion seedling survival (angular transformed data)
Black isolates were not significantly different (maximum overall l.s.d 5%: 11.4) from **Fus2 treatment** according to REML analysis
Grey isolates were significantly different (l.s.d 5%) from **Fo47 treatment** according to REML analysis
Blue isolates were not significantly different (l.s.d 5%) from **Fo47 treatment** and not significantly different (l.s.d 5%) from **Untreated** according to REML analysis (Di of Untreated was 57.54)
***overall maximum l.s.d. 5%**: approximate least significant differences (5% level) of REML means

The combined dataset was analysed in relation to host plants and results of the onion seedling assay (Figure 21). Eleven microsatellite haplotypes were exclusively found from onion (HT3, HT4, HT5, HT6, HT9, HT10, HT14, HT15, HT18, HT20 and HT21), carnation (HT2), freesia (HT1 and HT17), tomato (HT23), asparagus (ST16 and HT25), bunching onion (HT17), leek (HT8 and HT22), peas (HT24) and the biocontrol strain (HT12). On the other hand, a few sequence types were found in different formae speciales such as HT26 comprised formae speciales *cepae* and *gladiolus* isolates, HT13 included isolates from onion, garlic and tomato and HT19 consisted of isolates recovered from onion and garlic. The combined microsatellite dataset was analysed in relation to the results of onion seedling assay (Figure 21, Table 13). As previously, for interpretation of results isolates tested in the seedling assay were divided into arbitrary groups aggressive and non-aggressive based on statistical similarity to the Fo47 treatment.

Isolates representing 19 microsatellite haplotypes were exclusively found to be non-aggressive (HT1- to HT9, HT11, TH12, HT15- to TH17 and TH22- to HT26), while only four were considered as aggressive towards on onion (HT14, HT20, HT21 and HT22). Three haplotypes comprised both aggressive and non-aggressive isolates (HT10, HT13 and HT18). HT13 comprised isolates from tomato, garlic and onion and surprisingly the isolate from tomato was found to be aggressive, while one of the onion isolates was non-aggressive (Table 13). HT10 consisted of one non-aggressive and two aggressive onion isolates. HT18 contained an aggressive leek and a non-aggressive onion isolates (Table 13).

In summary, five published microsatellite markers were tested on the same set of isolates (40) as by housekeeping genes, and this led to the identification of 26

haplotypes (HTs). Unfortunately, only some of the microsatellite patterns could be linked to the pathogenicity e.g. HT14, HT20, HT21 and HT22.

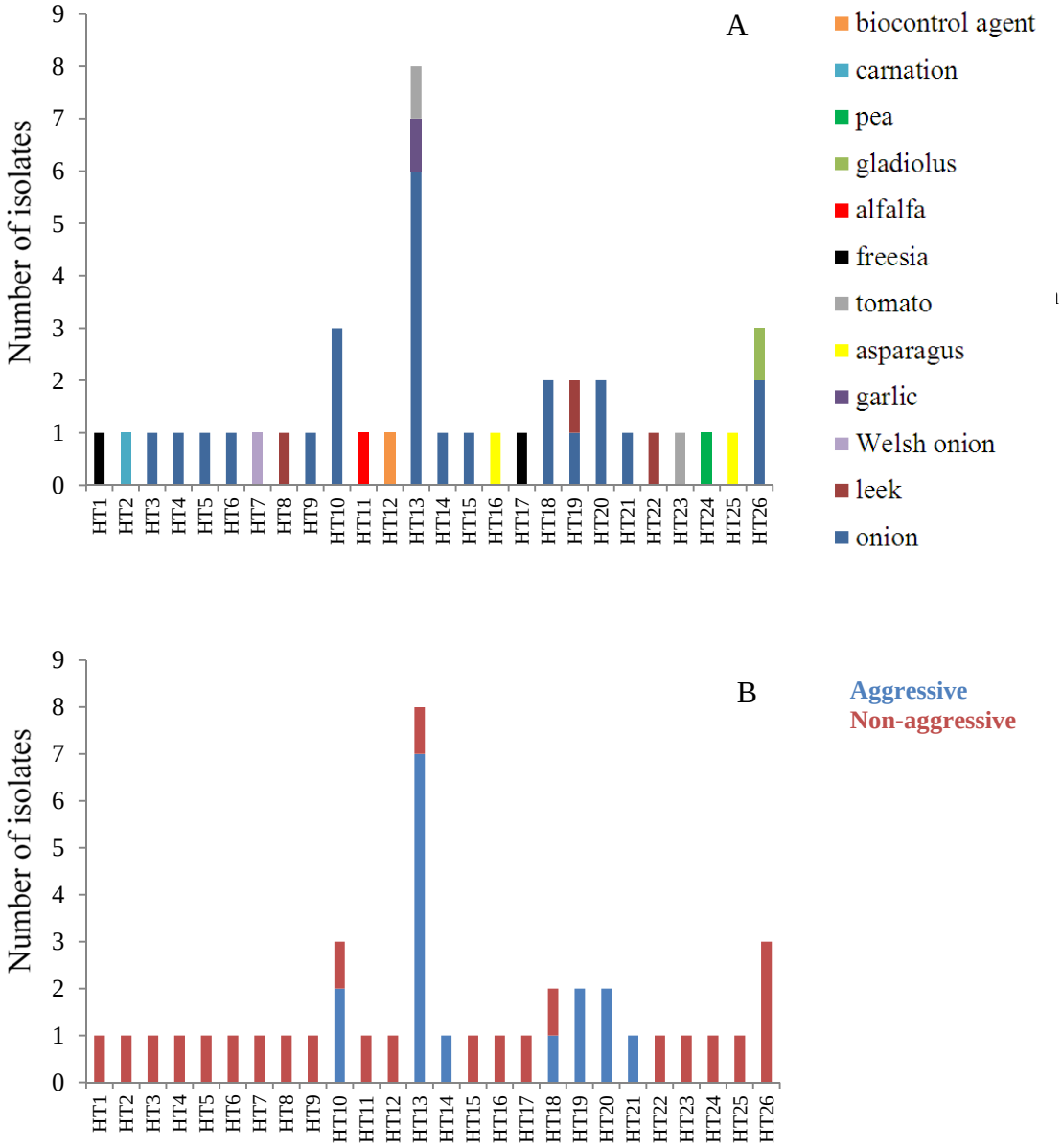


Figure 21. Frequency of microsatellite haplotypes (HTs) based on five published microsatellite markers (MB2, FOL680, FOL356, FOL35 and FOL245; Bogale *et al.*, 2005, Almany *et al.*, 2009) of 40 *Fusarium oxysporum* isolates from diseased *Allium* and other formae speciales for **A** different host plants and **B** relation to onion seedlings.

3.4.5 Development of microsatellite markers on a mobile pathogenicity chromosome of FOL for the characterisation of *F. oxysporum* isolates from diseased *Allium* species and different formae speciales

New microsatellite markers were developed and tested on a *SIX* gene encoding mobile pathogenicity chromosome (chromosome 14) of *F. oxysporum* f. sp. *lycopersici* (Ma *et al*., 2010; Broad Institute) to determine intraspecific variation within *F. oxysporum*.

Twelve loci were chosen for analysis and designed primers (Table 14) were initially tested on five *F. oxysporum* isolates representing formae speciales *cepae, lycopersici* and non-aggressive isolates to detect markers present in a wider range of formae speciales Only one marker, 21384, gave a single product, whereas the other markers gave no product (marker 2887) or multiple products (markers 20774 and 40094) or did not amplify from all five isolates (markers 8735, 4775, 48170, 10051, 83520, 68248, 38459 and 5465). Marker 21384 was chosen for further analysis, as it gave a product with all five isolates initially tested, although isolate D2 gave a faint band.

F. oxysporum isolates (36) representing several different formae speciales were chosen to be analysed by microsatellite marker 21348. The fragment length for marker 21348 varied between 120-187 bp (Appendix V). Seven isolates (including D2, A28, A5, FOA4, Gr4, NRRL 22538 NRRL 36311 and PR5) gave no product for marker 21384. Twenty-six isolates yielded products for both markers and were divided into haplotype groups (Table 15). Marker 21384 was informative, 11 unique haplotypes were found among 36 isolates, although seven isolates were not assigned into any haplotype groups.

Table 14. Microsatellite primer sequences, simple sequence repeat (SSR) motifs in the respective amplicons, number of repeats and location on chromosome 15 of *Fusarium oxysporum* f. sp. *lycopersici*.

Primer	Sequence	Contig	Start	Stop	SSR motifs	Repeats
8735F	CTGCGAGCAACGACCAAGT	51	8735	8744	CA	5
8735R	CAAGCGTCCAGTTGTCTCAGCG					
4775F	CTCACCGATCCTCACCGACCTC	51	4775	4806	ATTGA	6.4
4775R	CAGGCTTGCTAATCAATCAACG					
2887F	CACCCCAAATTAAATACAGCG	22	2887	2896	CA	5
2887R	GAAGTCGAGTCGAGAAACCTAC					
48170F	GCTGTTGAAGTTTCGCCAAAC	22	48170	48182	TA	6.5
48170R	GAGAACACCCTGAAGTGATC					
10051F	CACAGGTTTTCTTTTCTCGCG	22	10051	10089	TACA	9.75
10051R	GCTTTTCATGGCCCTGATTGAG					
20774F	GCTTGGTATAGTGGAGAACTC	22	20774	20785	CT	6
20774R	CAAAACCGCAAGCGTGCGAC					
83520F	GTATTAGTTCCTCAGAAGC	22	83520	83725	CATCCTCCTTTGA	15.84
83520R	CTTGGATACAACTGTGATCG					
68248F	GTATCAGGTTCATCGGTCTTAG	22	68248	68514	TCGGTCGTGTCGGTA	17.8
68248R	GTGTAAATGAACTATGGACTGC					
21384F-PET	GCGTAAAGTAAGTAGAACCAGAAG	36	21384	21396	AT	6.5
21384R	CTTGACTTGGTGGACGTCGATTTG					
40094F	CGGTGAGTACGGCGAGACTG	36	40094	40106	AG	6.5
40094R	GGACTTCAATTTCATTCTGTC					
38459F	GAGGATGACAGTGATAGCGAG	36	38459	38478	TTCC	5
38459R	CTTCGTCAATACACGCTATGC					
5465F	GGCAGGATACTGGACAAGAG	22	5465	5476	CT	6
5465R	GTAAATCTATTGGCAGTGTATTC					

The combined lineage specific microsatellite dataset was analysed in relation to host plants and results of onion seedling assay (Figure 22). Three haplotypes were exclusively found from onion (MPC1, MPC2 and MPC4), Welsh onion (MPC9), freesia (MPC8), carnation (MPC11) and tomato (MPC7). However, identical sequence types were found among various formae speciales. For instance MPC10 comprised formae speciales *cepae* and *lycopersici* isolates, MPC3 included isolates from onion and a biocontrol strain, MPC5 comprised isolates from onion, asparagus

and gladiolus whereas MPC6 consisted of isolates recovered from onion, leek, alfalfa and freesia.

Table 15. Microsatellite haplotypes for 36 *Fusarium oxysporum* isolates affecting various host plants using a single microsatellite marker (21384) designed on a mobile pathogenicity chromosome (MPC) of FOL with reference to survival index (SI), estimated repeat number and haplotypes.

Culture	Host	SI	Microsatellite - 21384 *	Haplotypes
A13	onion	**29.0**	1	MPC1
M9	onion	**30.4**	29	MPC2
N70_7	onion	**16.2**	29	MPC2
FO47	biocontrol agent	**38.3**	30	MPC3
SH1_1	shallot	**22.2**	30	MPC3
A1/2	onion	**21.4**	30	MPC3
SM105	onion	**40.5**	30	MPC3
151	onion	**34.0**	31	MPC4
FOA5	asparagus	**41.0**	33	MPC5
Fus1	onion	**32.3**	33	MPC5
NRRL26993	gladiolus	**41.7**	33	MPC5
18B	leek	**30.5**	39	MPC6
Fom004	alfalfa	**38.1**	39	MPC6
NRRL26990	freesia	**31.2**	39	MPC6
SM54	onion	**17.7**	39	MPC6
NRRL36425	tomato	**39.1**	45	MPC7
NRRL26988	freesia	**38.0**	62	MPC8
22	Welsh onion	**36.1**	64	MPC9
25	onion	**22.4**	65	MPC10
262	onion	**23.3**	65	MPC10
A14	onion	**16.0**	65	MPC10
A19	onion	**35.9**	65	MPC10
A35	onion	**22.7**	65	MPC10
FOB	onion	**29.7**	65	MPC10
Fus2	onion	**12.1**	65	MPC10
Fus3	onion	**19.7**	65	MPC10
NRRL22544	tomato (or onion)	**22.2**	65	MPC10
SIB	onion	**23.3**	65	MPC10
NRRL26222	carnation	**43.6**	119	MPC11
A28	onion	**34.6**	N.D.	
A5	onion	**37.4**	N.D.	
FOA4	asparagus	**41.0**	N.D.	
Gr4	garlic	**24.9**	N.D.	
NRRL22538	onion	**44.9**	N.D.	
NRRL36311	pea	**34.9**	N.D.	
PR5	leek	**39.5**	N.D.	

N.D: not detected

* Repeat number variation of 21384 is more likely to be the result of a deletion rather than variation in number of microsatellite repeats.

SI: survival index: percentage onion seedling survival (angular transformed data)

Black isolates were not significantly different (maximum overall l.s.d 5%: 11.393) from **Fus2 treatment** according to REML analysis

Grey isolates were significantly different (l.s.d 5%) from **Fo47 treatment** according to REML analysis

Blue isolates were not significantly different (l.s.d 5%) from **Fo47 treatment** and not significantly different (l.s.d 5%) from **Untreated** according to REML analysis (Di of Untreated was 57.54)

***overall maximum l.s.d. 5%**: approximate least significant differences (5% level) of REML means

The combined lineage specific microsatellite dataset was analysed in relation to the results of onion seedling assay (Figure 22, Table 15). As described previously, for interpretation, the results of the onion seedling assay were divided into arbitrary groups “aggressive” and “non-aggressive” based on statistical similarity compared to Fo47 treatment. Seven haplotypes were exclusively identified as non-aggressive (MPC1, MPC4, MPC5, MPC7, MPC8, MPC9 and MPC11), while none of the haplotypes were exclusively aggressive. Four haplotypes comprised both aggressive and non-aggressive isolates (MPC2, MPC, MPC6 and MPC10). MPC10 comprised isolates from onion and tomato (as previously mentioned the isolate from tomato was found to be aggressive) while one of the onion isolates was non-aggressive. MPC2 consisted of one non-aggressive and two aggressive onion isolates. MPC6 contained an aggressive onion and non-aggressive freesia, alfalfa and leek isolates. MPC3 comprised a non-aggressive onion isolate and biocontrol agent and two aggressive onion isolates.

In summary, twelve microsatellite markers encoded on a mobile pathogenicity chromosome of *F. oxysporum* f. sp. *lycopersici* (Ma *et al*., 2010; Broad Institute) were chosen for analysis and designed primers. After initial screening, one newly designed microsatellite marker (21384) was chosen for further analysis, which resulted in eleven haplotypes (MPC1-MPC11). Unfortunately, the new primers did not give products with all FOC isolates and none of the microsatellite patterns could be linked to the pathogenicity.

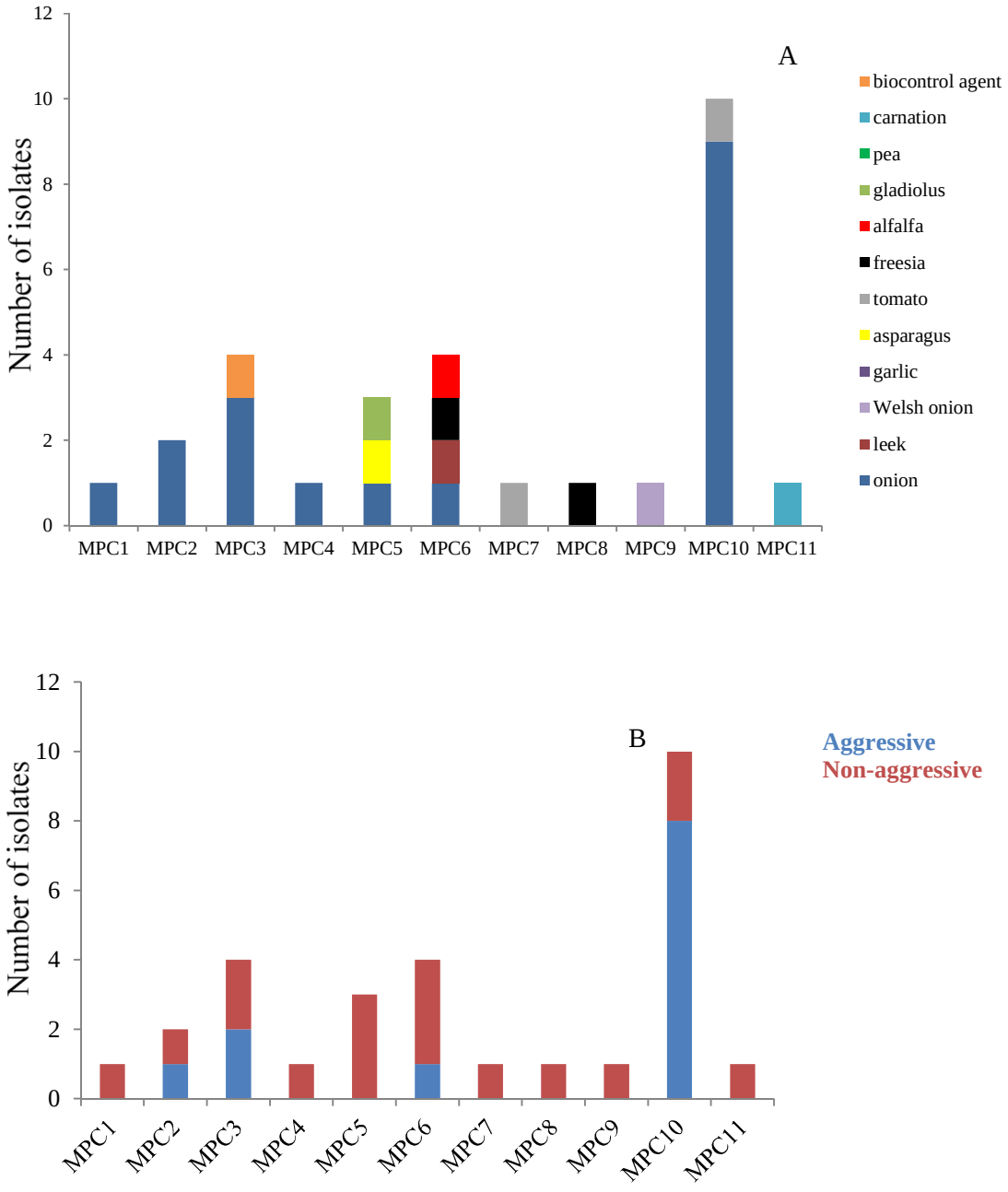


Figure 22. Frequency of microsatellite haplotypes based on a newly developed microsatellite marker (21384) of 36 *Fusarium oxysporum* isolates from diseased *Allium* and other formae speciales for **A** different host plants and **B** relation to onion seedlings.

3.4.6 Screening of published effector genes in *Fusarium oxysporum*

The screening of five *F. oxysporum* (ff. spp. *cepae, medicaginis, lycopersici, asparagi* and non-onion aggressive) isolates for the presence of seven effector genes led to the discovery of a homologue of the *SIX7* gene in the FOC Fus2 isolate. Homologues of six other effector genes were detected in other formae speciales, such as *SIX6* and *OXRED* in f. sp. *lycopersici*, *SIX8* in *lycopersici* and *medicaginis* and *FTF1* in *lycopersici, medicaginis* and *asparagi.*

F. oxysporum isolates (176) from diseased *Allium* crops were screened for the presence of *SIX7* homologue and compared with *TEF* sequence types (see Chapter 2; Appendix II, Figure 23). The homologue of *SIX7* was only present in the majority of isolates with sequence type FoA. Within *TEF* sequence type FoA, nine isolates tested negative and 128 tested positive for the presence of the *SIX7* gene. The nine isolates which tested negative were recovered from Australian leek (1 isolate), British garlic (2 isolates) and British onion (6 isolates).

When *F. oxysporum* isolates (23) representing twelve different formae speciales were also screened for the presence of *SIX7*, it was found in *F. oxysporum* ff. spp. *lycopersici* race1 and 2, *narcissi, gladioli and pisi* race 1 isolates, and was absent in formae speciales *tulipae, asparagi, medicaginis, freesia, dianthi,* and *pisi* race 2 and race 5. The *SIX7* PCR products were sequenced and compared to published reference sequences of formae speciales *lycopersici* and *lilii* (accession numbers: GQ268954 and GQ268960; Figure 24). All FOC isolates containing the *SIX7* homologue shared the same sequence (Figure 24; type SIX7_A). Surprisingly, the *SIX7* sequence of one of the *lycopersici* (NRRL22544) isolate was identical to the *SIX7* sequence of the *cepae* isolates.

Sequences of the other *lycopersici* isolates were identical (type SIX7_C) to the reference (accession number GQ268954). It has to be noted that the "*lycopersici*" isolate NRRL22544 was found to be aggressive in onion seedlings (Table 11). Sequencing of the *SIX7* homologues of formae speciales *narcissi* (type SIX7_B), *gladioli* (type SIX7_E) and *pisi* race 1 (type SIX7_F) isolates and comparison to reference isolates f. sp. *lilii* (type SIX7_D) revealed that there are differences between the formae speciales and that two separate clades were formed which may indicate specificity towards monocots or dicots, respectively (Figure 24).

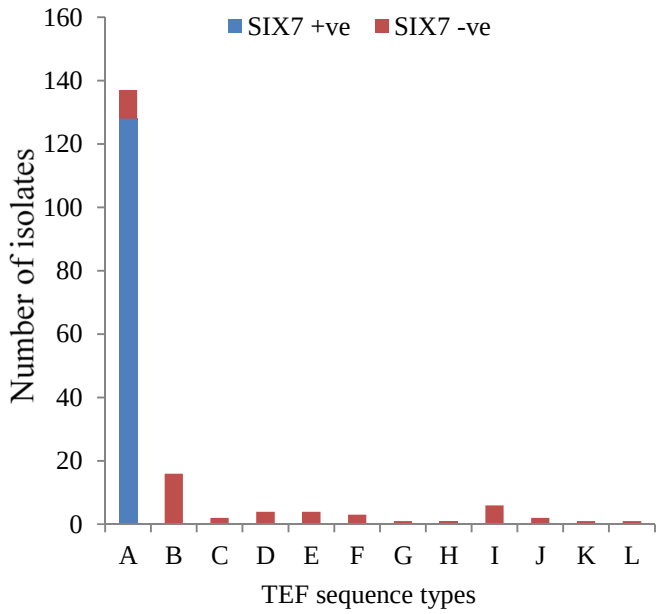


Figure 23. Frequency of *SIX7* gene among 176 *Fusarium oxysporum* isolates from *Allium* crops with reference to their TEF sequence type (FoA to FoL, see Figure 8).

In comparison with *F. oxysporum* f. sp. *lycopersici*, 91% DNA sequence identity was found for the *SIX7* homologue in *F. oxysporum* f. sp. *cepae*, 93% identity for *F. oxysporum* f. sp. *narcissi*, 95% identity for *F. oxysporum* f. sp. *pisi* race 1 and 89% identity for *F. oxysporum* f. sp. *gladioli*. A sequence alignment of representatives of ff. spp. *lycopersici, cepae, narcissi, lilii, pisi* race 1 and *gladioli*

also revealed that there are conserved and variable regions in the *SIX7* nucleotide sequence and in formae speciales *cepae, narcissi* a four bp long insertion causes a frameshift mutation whereas in the sequence of *F. oxysporum* f. sp. *gladioli* a 68 bp long deletion is present (Figure 25). *F. oxysporum* ff. spp. *lycopersici, narcissi, cepae* and *lilii SIX7* homologues were further analysed and it revealed that the proportion of replacement mutations are much higher when compared to silent mutations in the full coding sequence (675 bp) (Figure 25). For example, when FOC is compared to FOL there are two three bp long deletions (at positions 106-109 and 467-469) and a four bp long insertion (at position 494-497) which causes a frameshift mutation in the following sequence leading to stop codon at position 502-505. This could suggest that homologues *SIX7* gene of FOC encodes a shorter protein compare to FOL. The proportion of silent mutations *versus* replacement mutations before the 4 bp long insertion is 5 to 39 (S/R=5/39). This agrees with the protein sequence alignment of the four formae speciales mentioned above (Figure 26). Moreover, cloning of the *SIX7* gene into *E. coli* vectors and sequencing of clones confirmed that the insertion and all mutations are not artefacts of sequencing PCR reaction (data not shown).

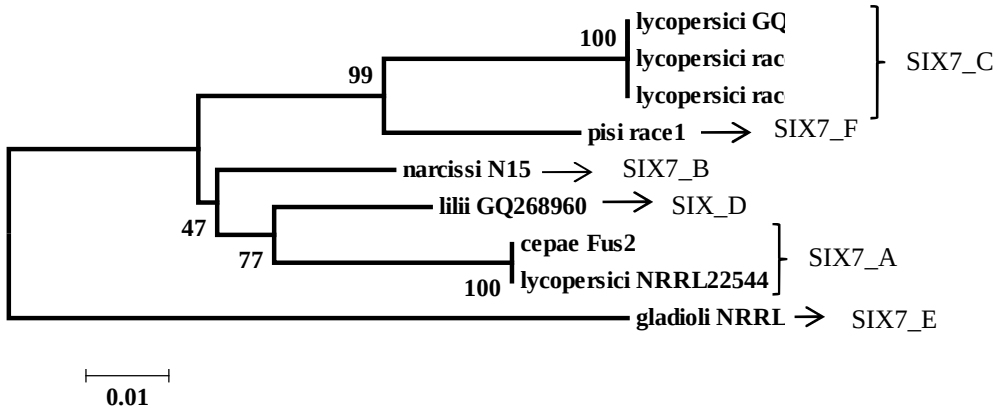


Figure 24. Cladistic tree of nine *Fusarium oxysporum* isolates belonging to six formae speciales based on "secreted in xylem 7" nucleotide sequences (*SIX7*). Consensus tree is shown with bootstrap values from 1000 replications. GenBank accession number is indicated for each reference sequence. SIX7_A- to SIX7_F refers to *SIX7* sequence types. NRRL 22544 was originally designated as *F. oxysporum* f. sp. *lycopersici*, but was found very aggressive in onion seedlings.

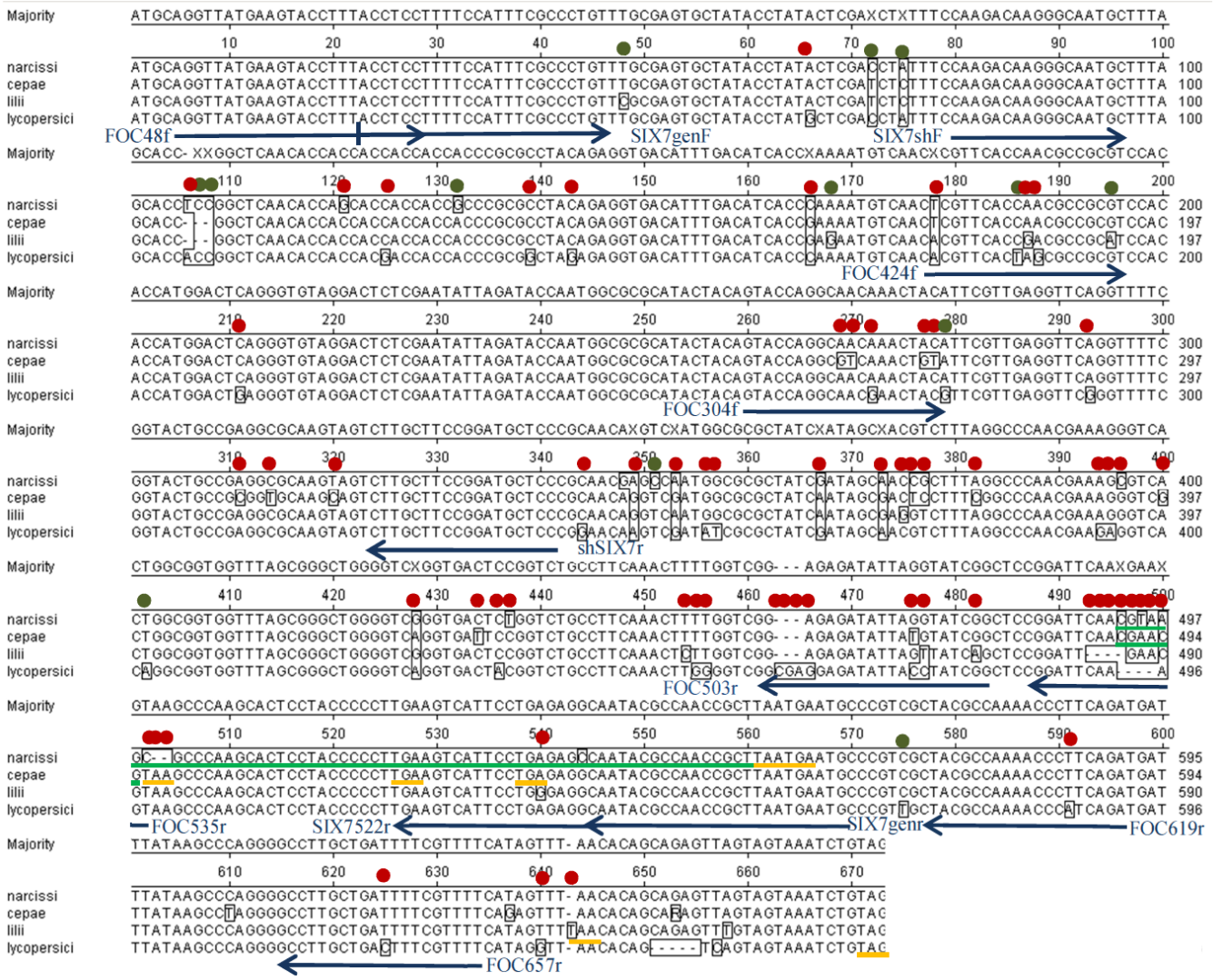


Figure 25. Multiple alignment of four nucleotide sequences: *SIX7* gene sequences of *Fusarium oxysporum* f. sp. *lycopersici* and three sequences corresponding to *SIX7* homologues of *F. oxysporum* ff. spp. *narcissi*, *cepae* and *lilii*. Differences compared to the consensus sequence are shown in boxes. Primer sequences are indicated with a blue arrow underneath the multiple alignment.
Silent mutations are indicated with a green spot above the multiple sequence alignment.
Replacement mutations* are indicated with a red dot above the multiple alignment.
Frame shift mutations* are underlined in green.
Stop codons are shown with a yellow line.
* Mutation in comparison to *F. oxysporum* f. sp. *lycopersici SIX7* gene.

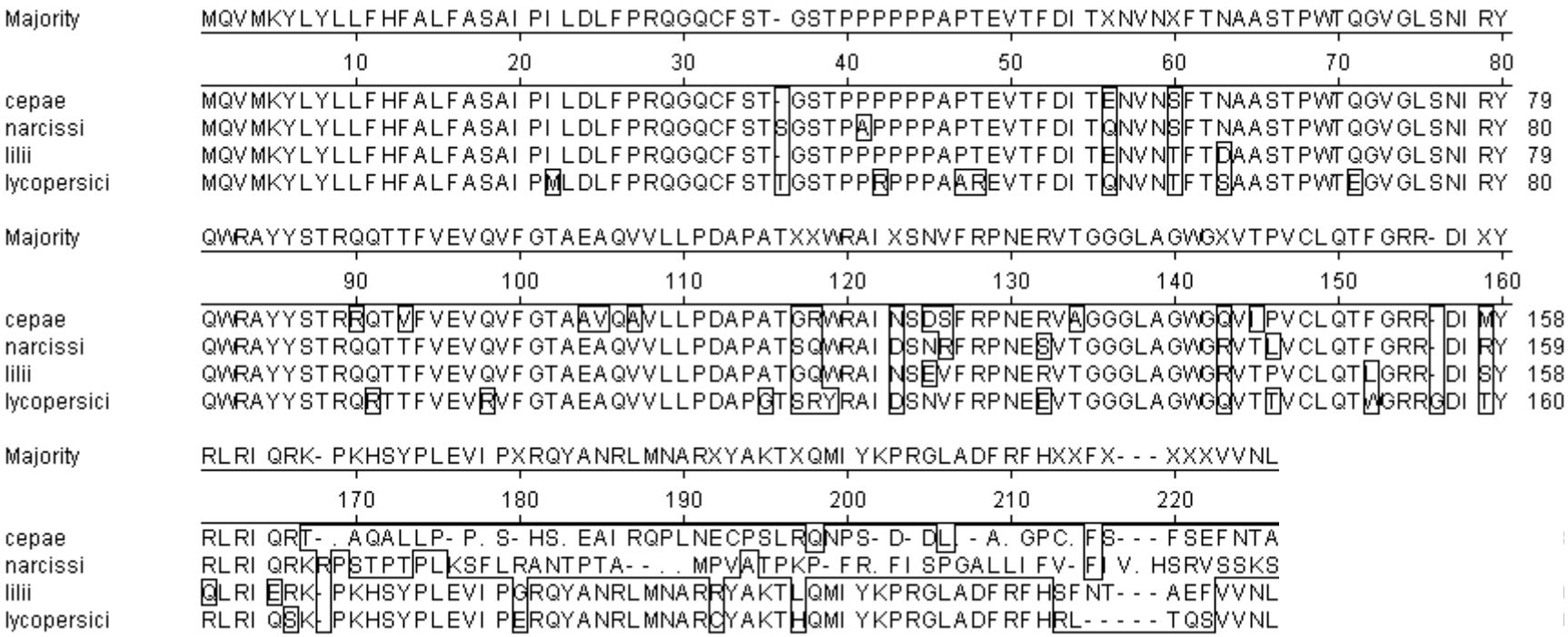


Figure 26. Multiple alignment of four protein sequences: *SIX7* gene sequence of *Fusarium oxysporum* f. sp. *lycopersici* and three sequences corresponding to *SIX7* homologues of *F. oxysporum* ff. spp. *narcissi*, *cepae* and *lilii*. Differences compare to consensus sequence are shown in boxes.

Primers (12) were designed on various regions of *SIX7* (Table 16) to confirm the presence / absence of the homologue of this gene in *F. oxysporum* ff. spp. *cepae* (Fus2, D2, GR4), *lycopersici* (FOLR1), *medicaginis* (Fom004) and *F. oxysporum* isolates tested non-aggressive in onion seedlings (A13, 151, A28; Table 17). None of the tested primer pair combinations (FOC403f/FOC535r, FOC403f/SIX7522r, FOC403f /FOC503r, FOC48f/FOC657r, FOC424f/FOC619r, SIX7genF/SIX7genR, SIX7shF/ SIX7shR; Tables 16 - 17) gave a product with D2 and Gr4 (very aggressive isolates). On the other hand, the shSIX7F/ShSIX7R primer pair (designed on the conserved region of *SIX7*) generated a band with the A13 isolate, which was found non-aggressive in onion seedlings. The A13 *SIX7* fragment was sequenced and it was identical to the ST SIX7_A. FOC-specific FOC304f and FOC353r primers gave a product only with FOC isolate Fus2 isolate, but were negative for *F. oxysporum* f. sp. *lycopersici* (FOLR1).

Table 16. Sequences of newly designed *SIX7* specific primers.

Primer	5' → 3' Sequence	Primer	5' → 3' Sequence
SIX7genF	CCTTTACCTCCTTTTCCATTTCGCCC	shSIX7f	CCAAGRCAAGGGCAATGC
SIX7genR	CATTCATTAAGCGGTTGGCGTATTG	shSIX7r	GGAGCATCCGGAAGCAAG
FOC619R	CATCTGAAGGGTTTTGGCGTAG	FOC424f	TCGTTCACCAACGCCGCG
FOC657R	GAAAACGAAAATCAGCAAGGC	FOC503r	CGATACATAATATCTCTCC
FOC48F	GGTTATGAAGTACCTTTACCTCC	FOC304f	ACCAGGCGTCAAACTGTA
SIX7522r	TGCCTCTCAGGAATGACTTCA	FOC535r	GCTTGGGCTTACGTTCGTTG

F or f: forward primer, R or r: reverse primer

Table 17. Results of PCR using seven primer pairs designed on *SIX7* homologue of *Fusarium oxysporum* ff. spp. *cepae*, *lycopersici* and *medicaginis.*

Isolates	FOC403f-FOC535r	FOC403f-SIX7522r	FOC304f-FOC503r	FOC48f-FOC657r	FOC424f-FOC619r	SIX7genF SIX7genR	SIX7shF SIX7shR
D2	negative	negative	negative	negative	M	negative	N.P.
151	negative	negative	negative	negative	M	negative	N.P.
A28	negative	negative	negative	negative	M	negative	N.P.
Fus1	negative	negative	negative	N.P.	M	N.P.	N.P.
Fus2	positive	positive	positive	positive	positive	positive	positive
FOLR1**	negative	negative	negative	positive	M	positive	positive
A13	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	positive
GR4	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	N.P.
Fom004*	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	N.P.

n.i.: no information, N.P: non-specific product, M: multiple products

*Fom004:*F. oxysporum* f. sp. *medicaginis*; **FOLR1: *F. oxysporum* f. sp. *lycopersici* race 1

The combined dataset of 40 *F. oxysporum* isolates was analysed in relation to presence/sequence of *SIX7* homologue and results of onion seedling assay (Table 18, Figure 27).

All non-A type *SIX7* isolates and A13 isolate carrying a truncated or mutated version of *SIX7* were exclusively found to be non-aggressive (Figure 27). Whereas isolates harbouring the A type of *SIX7* homologue and *SIX7* negative isolates could be aggressive or non-aggressive (Table 18, Figure 27). Aggressive isolates not carrying the *SIX7* homologue were recovered from onion, leek, shallot and garlic. Non-aggressive strains carrying type A sequence of the *SIX7* homologue were isolated from onion and leek (Table 18).

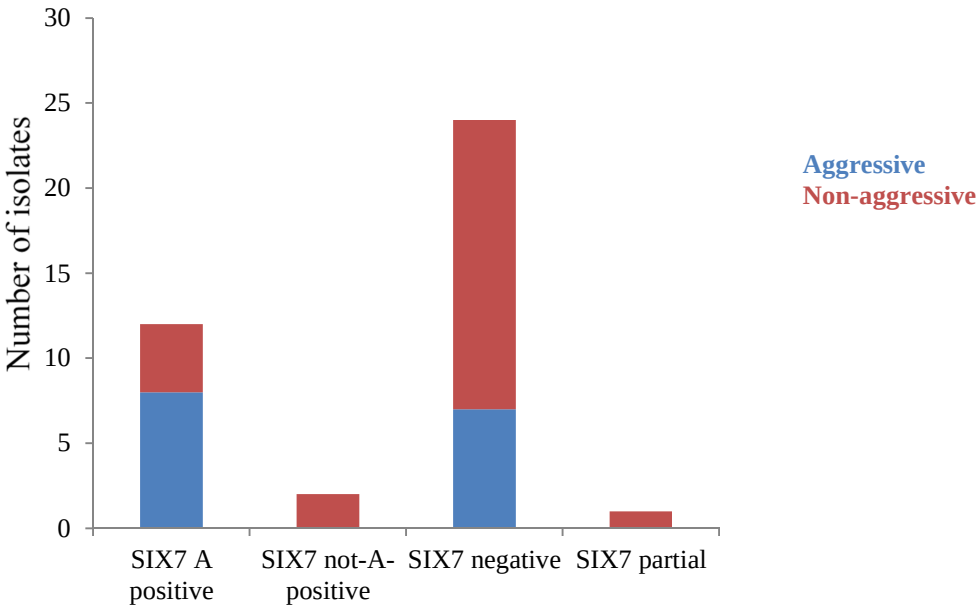


Figure 27. Distribution of SIX7_A and non-A type homologue of *SIX7* gene among 40 *Fusarium oxysporum* isolates in relation to results of onion seedling assay.

Table 18. Presence and sequence types of *SIX7* and for 40 *Fusarium oxysporum* isolates from diseased *Allium* hosts and other formae speciales.

Culture	Host	SI	SIX7	SIX7 sequence type
Fus2	onion	12.1	positive	SIX7_A
A14	onion	**16.0**	positive	SIX7_A
N70_7	onion	**16.2**	positive	SIX7_A
SM54	onion	**17.7**	negative	-
D2	onion	**18.2**	negative	-
PR7	leek	**19.1**	negative	-
Fus3	onion	**19.7**	positive	SIX7_A
180	onion	**21.2**	negative	-
A1/2	onion	**21.4**	negative	-
NRRL22544	tomato	**22.2**	positive	SIX7_A
SH1_1	shallot	**22.2**	negative	-
25	onion	**22.4**	positive	SIX7_A
A35	onion	**22.7**	positive	SIX7_A
262	onion	**23.3**	positive	SIX7_A
SIB	onion	**23.3**	positive	SIX7_A
Gr4	garlic	24.9	negative	-
A13	onion	29.0	partial	SIX7_A
FOB	onion	29.7	positive	SIX7_A
M9	onion	30.4	negative	-
18B	leek	30.5	negative	-
NRRL26990	freesia	31.2	negative	-
PG	onion	31.8	negative	-
Fus1	onion	32.3	negative	-
151	onion	34.0	negative	-
A28	onion	34.6	negative	-
NRRL36311	pea	34.9	negative	-
A19	onion	35.9	positive	SIX7_A
22	Welsh onion	36.1	negative	-
A5	onion	37.4	negative	-
NRRL26988	freesia	38.0	negative	-
Fom004	alfalfa	38.1	negative	-
FO47	biocontrol agent	38.3	negative	-
NRRL36425	tomato	39.0	positive	SIX7_C
PR5	leek	39.5	positive	SIX7_A
SM105	onion	40.5	negative	-
FOA4	asparagus	41.0	negative	-
FOA5	asparagus	41.0	negative	-
NRRL26993	gladiolus	41.7	positive	SIX7_E
NRRL26222	carnation	43.6	negative	-
NRRL22538	onion	44.9	negative	-

SI: survival index, percentage onion seedling survival (angular transformed data)
-: SIX7 not detected, SIX7_A to SIX7_E: SIX7 sequence types
Black isolates were not significantly different (maximum overall l.s.d 5%: 11.4) from **Fus2 treatment** according to REML analysis **Grey isolates** were significantly different (l.s.d 5%) from **Fo47 treatment** according to REML analysis **Blue isolates** were not significantly different (l.s.d 5%) from **Fo47 treatment** and not significantly different (l.s.d 5%) from **Untreated** according to REML analysis (Di of Untreated was 57.54)
***overall maximum l.s.d. 5%**: approximate least significant differences (5% level) of REML means

SUMMARY

A homologue of a previously published effector gene of FOL, *secreted in xylem 7* (*SIX7*), has been found in some of the FOC isolates, but not all of them. It was also shown that homologues of *SIX7* were only identified for some of the isolates when amplified by primers designed on conserved regions. Extensive PCR-based screening of 176 *F. oxysporum* isolates from *Allium* crops showing basal rot symptoms for the presence of *SIX7* homologues led to the discovery of *SIX7*'s limitation to one *TEF* ST of FOC, FoA. Genome sequencing of a very aggressive FOC isolate Fus2 led to the discovery of the homologues of further published effector genes, namely *SIX3, SIX5* and *SIX9*. This is the first report of the discovery of a homologue of *Fol-AVR2* (=*Fol-SIX3*) and *Fol-SIX5* in another forma specialis than FOL. Overall, *SIX3, SIX5* and *SIX7* are useful for the robust identification of some of the very aggressive FOC isolates, although some FOC isolates lack these genes.

In order to isolate further effector genes of *Fusarium oxysporum* f. sp. *cepae*, a whole genome sequencing approach was chosen and this work is discussed in Chapter 4.

3.5 DISCUSSION

Pathogenic and saprophytic *F. oxysporum* can co-exist in the same cropping system. Identification of *F. oxysporum* isolates at a sub-species level is essential for disease management purposes (Lievens *et al*., 2007). Onion basal rot in the UK is an increasing problem and it is mainly caused by FOC (Chapter 2 and Chapter 3). Prevention is the best way to control this soil-borne pathogen, which requires FOC-free onion seeds, sets and soil for crop production. The research in this chapter was focussed on genetic diversity in a collection of *F. oxysporum* isolates from *Allium* species using onion pathogenicity assay and molecular markers, such as housekeeping genes, microsatellite markers and effector genes to develop FOC-specific markers.

3.5.1 Cladistic analysis of *F. oxysporum* isolates associated with diseased *Allium* species and different formae speciales based on *TEF* sequences

FOC isolates showing diversity based on *TEF* sequence variation were analysed by neighbour-joining and maximum likelihood methods. Isolates formed two main clades, Clade 2 and Clade 3 having been determined by O'Donnell *et al*. (1998a), as supported by the position of isolates used in original study mentioned above. This result agrees with a previous study by Galvan *et al*. (2008) where a worldwide collection of 29 FOC isolates was analysed using AFLP markers. Isolates classified as belonging to sequence types (STs) FoA, FoB and FoD cluster closely, suggesting a clonal origin of these groups, although these clonal lineages were identical to representatives of other formae speciales based on partial *TEF* sequence similarity. For example, the most commonly occurring clonal lineage FoA comprises

identical *TEF* sequences of cultures pathogenic to *Allium* species, tomato, peas, freesia alfalfa and asparagus. The diversity of isolates from *Allium* species indicates the polyphyletic origin of FOC. This finding agrees with previous research, several formae speciales such as *cepae*, *lycopersici*, *freesia*, *pisi*, *asparagi*, *medicaginis*, *gladioli* and *dianthi* were described as polyphyletic (Galvan *et al*., 2008; Baayen *et al*., 2000).

3.5.2 Onion seedling assay using *Fusarium* isolates from diseased *Allium* species and different formae speciales

Classically, the identification of host specific forms of *F. oxysporum* is done by time-consuming and laborious pathogenicity assays. Moreover, pathogenicity testing does not give information on host specificity of strains determined as non-pathogenic towards a few tested plants (Leslie and Summerell, 2006).

A previously developed seedling assay (Taylor *et al*., 2012) was used in this study to characterise *Fusarium* isolates associated with onion basal rot. This assay was chosen because it provides relatively rapid results that are consistent with disease severity observed using a more laborious onion bulb rot assay. Taylor *et al*. (2012) compared both assays using under the same glasshouse conditions. In the bulb rot assay, onion sets were grown in *Fusarium* infested soil for six months, and observed basal rot symptoms (pink mycelium development on basal plate and browning scales) in the susceptible control. The seedling assay was completed in four weeks, and gave consistent results with less expensive and labour, which is very important for large-scale characterisation of *Fusarium* isolates and onion cultivars.

There was a significant variation in aggressiveness among *F. oxysporum* isolates from different sources and locations in the UK. These results agree with previous studies from Japan (Dissanayake *et al.*, 2009ab) where 13 *F. oxysporum* isolates were tested on Welsh onion seedlings and were found to be strongly, moderately and non-aggressive.

The results also confirmed that *F. proliferatum* was pathogenic on onion, although there was variation in pathogenicity. *F. solani* and *F. redolens* were found on onions grown in the UK as frequently as *F. proliferatum*, but the results of the pathogenicity assay suggested that they are not causal agents of onion basal rot. *F. redolens* was tested as non-aggressive in an onion seedling assay in Japan (Dissanayake *et al*., 2009ab). On the contrary, *F. solani* and *F. redolens* isolates from onion were found to be pathogenic in Turkey (Bayraktar *et al*., 2010). The simplest explanation for this difference is that the *F. solani* and *F. redolens* isolated from British onion occur as secondary invaders rather than as pathogens. However, only two *F. solani* and four *F. redolens* isolates were included in our pathogenicity assay and these could be atypical. This disagreement could also be explained by the lack of negative standard (*e.g*. non-aggressive Fo47 strain) in assays employed by other research institutes, where only water was used as negative control. Results of the seedling assay using Spanish *F. proliferatum* and *F. acuminatum* isolates showed that they can cause damping-off symptoms (Palmero *et al*., 2012), which also agrees with a previous publication from Turkey (Bayraktar *et al*., 2010). Prior evidence in the literature for the presence of *F. proliferatum* and *F. acuminatum* in association with onion basal rot in Spain and Chile was not found. One of the *F. oxysporum* isolates from the Dutch set was found to be very aggressive in onion seedlings. This suggests that FOC can spread by infected planting material between countries.

3.5.3 Molecular identification of *F. oxysporum* isolates using DNA sequence variation in housekeeping genes

Four housekeeping genes *TEF*, calmodulin, B-tubulin and *RPB2* were used for the characterisation of 40 *F. oxysporum* strains representing *F. oxysporum* ff. spp. *cepae, lycopersici, pisi, medicaginis, asparagi, freesia, dianthi, gladioli* along with non-aggressive isolates recovered from onion and biocontrol strain Fo47. The combination of the four datasets led to the identification of 19 sequence types (STs). Two of these (ST4 and ST8) correlated with aggressiveness in onion seedlings. However, four sequence types (STs 1- to 3 and ST6) comprised both aggressive and non-aggressive isolates. Within these sequence groups, all isolates recovered from non-*Allium* hosts were found non-aggressive in onion seedlings, except for isolate NRRL22544 which was misidentified as belonging to *F. oxysporum* f. sp. *lycopersici*. *TEF, RPB2*, B-tubulin and calmodulin genes have not been used together to characterise pathogen populations. However, 18 formae speciales were characterised by β-tubulin, *TEF*, microsatellites and AFLP, but none of these markers were able to differentiate representatives of different host-specific forms of *F. oxysporum* (Bogale *et al*., 2006). *RPB2* was first used to characterise *Fusarium* pathogen populations in a study based on 126 clinically important fusaria (O'Donnell *et al*., 2007). However, *RPB2* has not been used before to characterise *F. oxysporum* representing various formae speciales *RPB2* and *TEF* were more informative compared to calmodulin and β-tubulin, and therefore more appropriate for phylogenetic studies, as suggested by other researchers (Poucke *et al*., 2012).

3.5.4 Molecular identification of *F. oxysporum* isolates using microsatellite markers

Microsatellite markers provide a powerful tool to study taxonomy and population genetics (Jimenez-Gasco *et al*., 2003). In this study, five previously published microsatellite markers were tested to determine the intraspecific variation within the *F. oxysporum* species complex. In total, 26 unique haplotypes were found among 40 *F. oxysporum* isolates representing various formae speciales FOL 356 and FOL 245 were found more informative than MB2 and FOL35, while FOL680 did not show a high level of variation. Loci FOL 35 and FOL 680 had the highest diversity in *F. oxysporum* ff. spp. *lycopersici* and *radicis-lycopersici* (Almany *et. al*., 2009), while loci MB2 showed the highest diversity of isolates from Ethiopian soil and plant tissue (Bogale *et al*., 2005). A Blast search of microsatellite targeting primers revealed that none of these markers are present on lineage specific regions.

Some of the microsatellite patterns could be linked to aggressiveness in onion seedlings (*e.g*. HT14, HT20, HT21 and HT22). However, three haplotypes comprised both aggressive and non-aggressive isolates: HT10, HT13 and HT18. HT13 comprised the misidentified *F. oxysporum* f. sp. *lycopersici* isolate NRRL22544. Apart from this, all other isolates were recovered from *Allium* species regardless of their aggressiveness in onion seedlings (Table 13). This means that microsatellites were not useful to distinguish aggressive and non-aggressive isolates of *Fusarium oxysporum*, which was also found by Bogale *et al*. (2006). However, if the onion isolates that tested non-aggressive in HT10, HT13 and HT18 (isolates A19, FOB and 151) are atypical due to cultural instability caused by repeated sub-culturing, in that case the combination of five microsatellites tested enables us to

identify *F. oxysporum* f. sp. *cepae* (Leslie and Summerell, 2006; de Visser and van den Broek, 2005), although this is just speculation.

3.5.5 Molecular identification of *F. oxysporum* isolates using microsatellite markers designed on mobile pathogenicity chromosome of FOL

Lineage specific regions and chromosomes in the genome of *F. oxysporum* f. sp. *lycopersici* 4287 were directly linked to pathogenicity towards tomato (Ma *et al*., 2010). The objective of this small study was to develop microsatellite markers on these lineage specific regions for the characterisation of the *F. oxysporum* species complex.

Several microsatellite markers were developed on supercontig 36 of *F. oxysporum* f. sp. *lycopersici* strain 4287 (FOL4287) harbouring some of the virulence genes. Unfortunately none of these markers gave products with all FOC isolates. This could mean that the different clonal lineages of FOC share different pathogenicity chromosomes. Those isolates that gave PCR products with primers designed on lineage specific region of FOL4287 carried similar lineage specific regions to the tomato pathogens. The comparative study of 11 plant pathogenic *F. oxysporum* strains representing eight formae speciales showed that genome-wide rearrangements can happen in a short evolutionary divergent time and this genomic plasticity that enables organism to adapt to their hosts (presentation by Dr Li Jun Ma at the ECFG11 conference, Marburg, Germany 2012).

3.5.6 Molecular identification of *F. oxysporum* isolates using effector genes

Identification of *F. oxysporum* ff. spp. *lycopersic*i (FOL) and *vasinfectum* based on effector genes was found to be a very robust identification method (Lievens *et al*., 2009a; Chakrabarti *et al*., 2011). It was also discovered that some of these effector genes are not restricted to only one formae speciales (Lievens *et al*., 2009a). Therefore, the aim of this study was to screen FOC isolates for the presence of previously published effectors and use it to develop specific primers.

Screening of seven effector genes led to the discovery of homologues of *SIX7* gene in Fus2 isolate belonging to FOC. A homologue of *SIX7* was previously found in *F. oxysporum* f. sp. *lilii* (Lievens *et al*., 2009a). Homologues of *SIX6, SIX8, OXRED* and *FTF1*genes were absent in isolate Fus2 using the primer pairs employed in the current study. It is likely that homologues of these genes are actually present but due to mutations in the primer annealing site, they were not detected. It was also shown that homologues of *SIX7* were only identified for some of the isolates when amplified by primers designed on conserved regions.

Extensive screening of 176 *F. oxysporum* isolates from *Allium* crops for the presence of *SIX7* homologues led to the discovery of *SIX7*'s limitation to one clonal lineage of FOC. The homologue of *SIX7* was only present in the most representative *TEF* sequence type FoA, but not in the other *TEF* STs. Screening of twelve other formae speciales led to the discovery of homologues of *SIX7* in *F. oxysporum* ff. spp. *narcissi*, *gladioli* and *pisi* race 1. Surprisingly, no *SIX7* genes were found in *F. oxysporum* f. sp. *gladioli* by Lievens *et al*. (2009). Comparison of the sequences of *SIX7* homologues revealed that they evolve faster compared to housekeeping genes, possibly due to positive selection. The *SIX7* gene of FOC is strongly conserved

among the representatives of this f. sp., which agrees with previous research (Lievens *et al*., 2009a; Thatcher *et al*., 2012).

Remarkably, the *SIX7* sequence of the NRRL22544 isolate was identical to the sequence of FOC isolates which agrees with the results of the onion seedling assay, therefore it is likely that this isolate was misidentified as belonging to FOL. To confirm this, host specificity of NRRL22544 needs to be tested on tomato as well as onion. This finding shows there is a need for reliable identification methods to distinguish formae speciales.

Primer pairs designed on the conserved regions of *SIX7* revealed the presence of a mutated or truncated version of this gene in a non-aggressive isolate from onion (A13). This finding is supported by the results of sequence comparison of *SIX7* homologues. The *SIX7* protein is likely to be shorter due to frameshift mutation when compared to the one in FOL. Moreover, primer pairs designed on the conserved region of the *SIX7* gene can be used for the reliable screening for the presence of this gene. On the other hand, primers designed on the variable regions of *SIX7* can be used to predict host specificity of several formae speciales. Unfortunately, *SIX7* is not present in all FOC isolates therefore it is not applicable to identify all onion-specific *F. oxysporum* isolates. However, the homologue of *SIX7* is present in the majority of FOC isolates in our collection, which suggest that it could be still valuable for identification methods using multiple loci. Surprisingly, two isolates (A19 and FOB) tested as non-pathogenic harboured *Foc-SIX7* gene, which suggests that these isolates may have lost their pathogenic ability due to cultural instability or epigenetic differences (Leslie and Summerell, 2006; Michielse *et al*., 2009, de Visser and den Broek, 2005).

One explanation for the lack of *SIX7* in some FOC isolates could be that the loss of *SIX7* is advantageous to overcome plant immunity (Takken and Rep, 2010). If the Six7 protein is recognised by *Allium* plants, FOC might have tried to escape recognition by losing *SIX7*. For example, FOL race 1 isolates differ from FOL race 2 and FOL race 3 isolates in the presence of an effector gene, *SIX4* (=*AVR1*), which was lost from FOL race 2 and FOL race 3 to avoid recognition by tomato plant immunity (Houterman *et al*., 2008; Takken and Rep, 2010). The other strategy to overcome plant immunity is by mutating effectors in order to avoid recognition as it happened in FOL; *Fol-SIX3* of FOL race 2 differs in two amino acids from FOL race 3 isolates. It has to be noted that no races have been discovered within FOC yet; therefore it is only a hypothesis. Moreover, it is also possible that the *SIX7* homologue of FOC is not functioning, due to the missense mutation, therefore it could become dispensable. Another hypothesis is that *SIX7* is not crucial for pathogenicity and it is located in a region of a chromosome be easily lost, or else *SIX7* was gained by these strains. This suggests that there is another set of effector gene(s) which are involved in host specificity and pathogenicity in FOC. This predicted gene(s) could be used for robust identification of *F. oxysporum* pathogenicity towards onion.

CHAPTER 4

GENOME SEQUENCING OF A *FUSARIUM OXYSPORUM* F. SP. *CEPAE* ISOLATE AND IDENTIFICATION OF CANDIDATE EFFECTOR GENES

4.1 INTRODUCTRION

This chapter focuses on the possible ways of identifying candidate effector genes applicable for the development of a *F. oxysporum* f. sp. *cepae* (FOC)-specific molecular methodology. A whole genome sequencing approach was chosen to identify candidate effector genes in the genome of a FOC isolate. This only provides preliminary results and future prospective are discussed.

4.1.1 Definition of effectors

Effectors can be defined as small secreted proteins that alter host cell structure and function, facilitate infection and suppress or activate effector-triggered immunity (van de Wouw and Howlett, 2011). Kamoun (2009) defined effectors as "molecules that alter host cell structure and function, thereby facilitating infection (*i. e.* virulence factors or toxins) and/or triggering defence responses (*i.e.* avirulence factors or elicitors)". Effectors can also be defined as "secreted proteins and other molecules which allow plant-associated organisms to modulate plant defence circuitry and enable colonization of plant tissue" (Hogenhout *et al*, 2009). Brown and his colleagues (2012) expanded that 'effectors' that modulate the interaction between pathogenic microbes and hosts have been identified from all (mutualistic, biotrophic, hemibiotrophic, necrotrophic and saprophytic) lifestyles".

In this chapter the definition of effectors is used as by Hogenhout *et al*. (2009). Characteristics listed in the first chapter (see Section 1.9) could be useful to discover / identify novel effector genes applicable for the development of specific molecular methods.

4.1.2 Characteristics of known *F. oxysporum* effectors

This section is predominantly focusses on effectors occur in *F. oxysporum*. However, characteristics of other fungal and oomycete effectors, listed in Chapter 1, need to be considered for the identification of novel candidate effectors.

Effector proteins can be classified based on whether they interact with their target in the host apoplast or cytosol (Kamoun, 2008). *SIX* genes of *F. oxysporum* are secreted during infection into the host apoplast, and are generally small (<300 amino acids), cysteine rich (two or more) proteins with a type II secretion signal at the N-terminal (Rep, 2005; van der Does and Rep, 2007). *SIX* genes have no database representatives outside of the genus *Fusarium* (Houterman *et al*., 2007), and the only sequence similarity between them was found in their promoter region, where a transposable element, mimp1, is harboured in all *SIX* genes (Sarah Schmidt, University of Amsterdam, pers. comm.).

Almost all known *SIX* genes of *F. oxysporum* f. sp. *lycopersici* (FOL) are coded on a mobile, lineage specific chromosome (chromosome 14) which is rich in transposable elements (Ma *et al*., 2010). Similarly, the first conditionally dispensable chromosomes encoding genes that are required for pathogenicity on pea, such as *pea pathogenicity* (*PEP1*, *PEP2*, and *PEP5* genes) and the *pisatin demethylase* (*PDA1*) gene, were discovered in the genome of *Fusarium solani* f. sp. *pisi* (Han *et al*. 2001).

The FOL effector gene *SIX1* (termed *Fol-SIX1*) is highly up-regulated during colonisation and requires living plant cells as only 5–20% of hyphae express *Fol-SIX1* when growing in tomato cell cultures (van der Does *et al*., 2008). Expression of the *Fo5176-SIX4* homologue of the *Arabidopsis* pathogen *F. oxysporum* was 18- to 70- fold less when grown on PDA plates compare to that measured *in planta* (Thatcher *et al*., 2012). In some fungi, effectors are highly expressed during nitrogen

starvation (Snoeijers *et al*., 1999, Stephenson *et al.,* 2000; Talbot *et al*., 1993) although in *F. oxysporum,* no up-regulation of effector genes was detected during nitrogen starvation of FOL (Divon *et al*., 2005), but a very low secretion was observed when the fungus was grown on minimal medium with sucrose and KNO_3 (van der Does and Rep, 2007).

Recently, two possible effector encoding genes, *putative effector protein 1* (*Fov-PEP1*) and *Fov-PEP2*, have been discovered from *F. oxysporum* f. sp. *vasinfectum* (FOV) (Chakrabarti *et al*., 2011). These genes were identified in a cDNA library of 2100 clones isolated from cotton seedlings infected with FOV under tissue-culture conditions (McFadden *et al*., 2006) and selected on the basis of criteria such as short, cysteine rich, secreted, had no database representatives at all or outside of *Fusarium* genus and was only present in FOV isolates. A homologue of *Fov-PEP1* is present in the FOL genome sequence as a single copy gene on chromosome 14, interrupted by a retrotransposon, while *Fov-PEP2* had no homologues in the FOL genome at all.

4.2 AIMS AND OBJECTIVES

The aims of this part of the project were:

- To obtain the whole genome sequence of a *F. oxysporum* f. sp. *cepae* (FOC) to identify candidate effector genes in a representative isolate
- To screen a set of candidate effector genes in a collection of *F. oxysporum* isolates representing several formae speciales for the presence / absence.
- To compare the sequences of putative effector genes from different formae speciales
- Evaluate and test bioinformatics tools and markers for the identification of further effector genes

4.3 MATERIALS AND METHODS

4.3.1 Preparation of DNA samples for genome sequencing of *F. oxysporum* f. sp. *cepae* (FOC)

4.3.1.1 Description of FOC isolate chosen for genome sequencing

A very aggressive FOC strain, Fus2, was used for whole genome sequencing. Fus2 was isolated from onion grown in Lincolnshire, UK and has been used to screen onion cultivars for resistance and also to test effectiveness of biocontrol treatments (Taylor *et al*., 2012; Ralph Noble, East Malling Research, pers. comm.).

4.3.1.2 DNA extraction

The CTAB extraction method (Li *et al*, 1994 modified by Dez Barbara, University of Warwick) was employed to obtain good quality (unsheared) DNA for genome sequencing. 250 mg of freeze dried mycelium of Fus2 was homogenised with 1 g of sterile sand using a chilled, sterile pestle and mortar. The ground mycelium/sand was transferred into a 50 ml Falcon tube and mixed by inversion with 15 ml extraction buffer (8.18g NaCl dissolved in 70 ml dH_2O with 5 ml 2M pH 8.0 Tris-Cl, 4 ml 0.5M pH 8.0 EDTA, 7.4 ml 10% cetyltrimethylammonium bromide (CTAB), 13.6 ml dH2O, 2 g polyvinylpyrrolidone 40 (PVP-40) and 0.5 ml 2-mercaptoethanol). The sample was incubated at 60°C for 30 minutes and mixed gently by inverting occasionally. 15 ml of the chloroform/penta-1-ol wash (96 ml chloroform and 4 ml penta-1-ol) was added to the sample which was mixed by gentle inversion for 10 minutes followed by centrifugation. All centrifugation steps were carried out at 1500 g (2914 rpm) for 10 minutes at 20°C, unless stated otherwise. The

aqueous phase was transferred into a sterile 50 ml Falcon tube and 15 ml of chloroform/penta-1-ol wash was added again followed by gentle mixing by inversion for 10 minutes then centrifuged. The aqueous phase was transferred into a sterile 50 ml Falcon tube and centrifuged again. 0.6 volumes of cold (-20°C) isopropanol was added to aqueous phase and mixed by inverting tube gently. The sample was incubated at room temperature for 2 hours before centrifuging at 460g (1500 rpm) for 2 minutes at 20°C. The supernatant was then removed and the pellet dissolved in 10 ml wash buffer (66 ml 100% ethanol and 34 ml 0.1M NaCl) by gentle inversion for 20 minutes followed by centrifugation. Supernatant was removed and rinsed again in 10 ml wash buffer then was centrifuged. The supernatant was removed and the pellet was air dried for 20 minutes. The pellet was then dissolved in 1.5 ml Tris-EDTA (10mM pH 8.0 Tris-Cl and 1 mM EDTA) and transferred into a 1.5 ml Eppendorf tube using a pipette tip with the end cut off. The sample was centrifuged at maximum speed for 5 minutes and the supernatant was transferred into a 1.5 ml Eppendorf tube using a pipette tip with the end cut off. The sample was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific).

4.3.1.3 RNase treatment and purification of the nucleic acid extract

RNase treatment was applied to degrade any RNA in the sample. Approximately 10µg of DNA was taken and 1 µl of RNase (Qiagen RNase A 7 U/µl) added in a total volume of 25 µl. The sample was incubated at room temperature for 30 minutes then desalted using a Qiaex II gel extraction/desalting kit (Qiagen) following the manufacturer's guidelines with a minor modification. The sample was eluted once in 30 µl of buffer EB from a QIAQuickPCR purification kit (Qiagen). The sample was quantified using a Nanodrop 1000 spectrophotometer (Thermo

Scientific) then by Qubit fluorospectrometer using a broad range dsDNA kit (Invitrogen). The DNA was diluted using buffer EB (Qiagen) to give a final volume of 75 µl and a concentration of 20 ng/µl.

4.3.1.4 Verification of purity and quality of the DNA sample

Amplification of the ITS region (using ITS1 and ITS4 primer pair and conditions published by White *et al*., 1990) and the *TEF* gene (Chapter 2) were used to ensure that the DNA sample is not contaminated and applicable for amplification.

4.3.2 Preparation of genomic DNA libraries and genome sequencing

Clone libraries containing 300-400 bp fragments were prepared by Jeanette Selby (Genomics Facility of University of Warwick) following Illumina's TruSeq DNA Sample Preparation low throughput (LT) protocol. Samples were run on an Illumina GAIIx sequencing platform which resulted in 70 bp long paired-end reads in fastq file format.

4.3.3 *In silico* analysis pipeline to identify putative effectors genes of Fus2

An *in silico* analysis pipeline was designed to identify putative effectors from the genome of Fus2 (Figure 28). Two approaches were chosen to assemble the 70 base pair long paired-end reads: template based assembly followed by *de novo* assembly of unused reads and *de novo* assembly. The first method was template based assembly using the *F. oxysporum* f. sp. *lycopersici* 4287 (FOL4287) genome and mitochondrial sequence (Broad Institute) as a reference followed by *de novo*

assembly of non-matching reads (see 4.3.3.1). The second method was *de novo* assembly utilizing all reads. Obtained contigs were screened for the presence of published effector genes (see 4.3.3.1). To identify candidate effector genes two approaches were taken. Firstly, contigs obtained through template based assembly followed by *de novo* assembly were appointed to gene prediction. Secondly, contigs obtained by *de novo* assembly and harbouring published effectors were chosen for further analysis. Characteristics of predicted genes were analysed by using various software packages and web servers (see below).

4.3.3.1 Assembly of paired-end reads

Velvet (Zerbino & Birney, 2008) was used for the *de novo* assembly of all reads. This method allows the formation of contigs based purely on their similarity to each other. This work was carried out by Dr Richard Harrison (East Malling Research).

Assembly of 70 base pair long paired-end reads by SeqMan NGen (DNAStar) using FOL4287 and mitochondrial genome (Broad Institute) as a template was employed for the identification of genomic regions highly similar to the template. Reads showing no similarities FOL4287 were not used for the template based assembly / alignment (e.g. unused reads). These unused reads were presumed to contain reads specific to the FOC genome. Unused reads were appointed to a *de novo* assembly using Geneious Pro (Drummond *et al.*, 2011; Biomatters Limited). This work was done by Riccardo Baroncelli (University of Warwick).

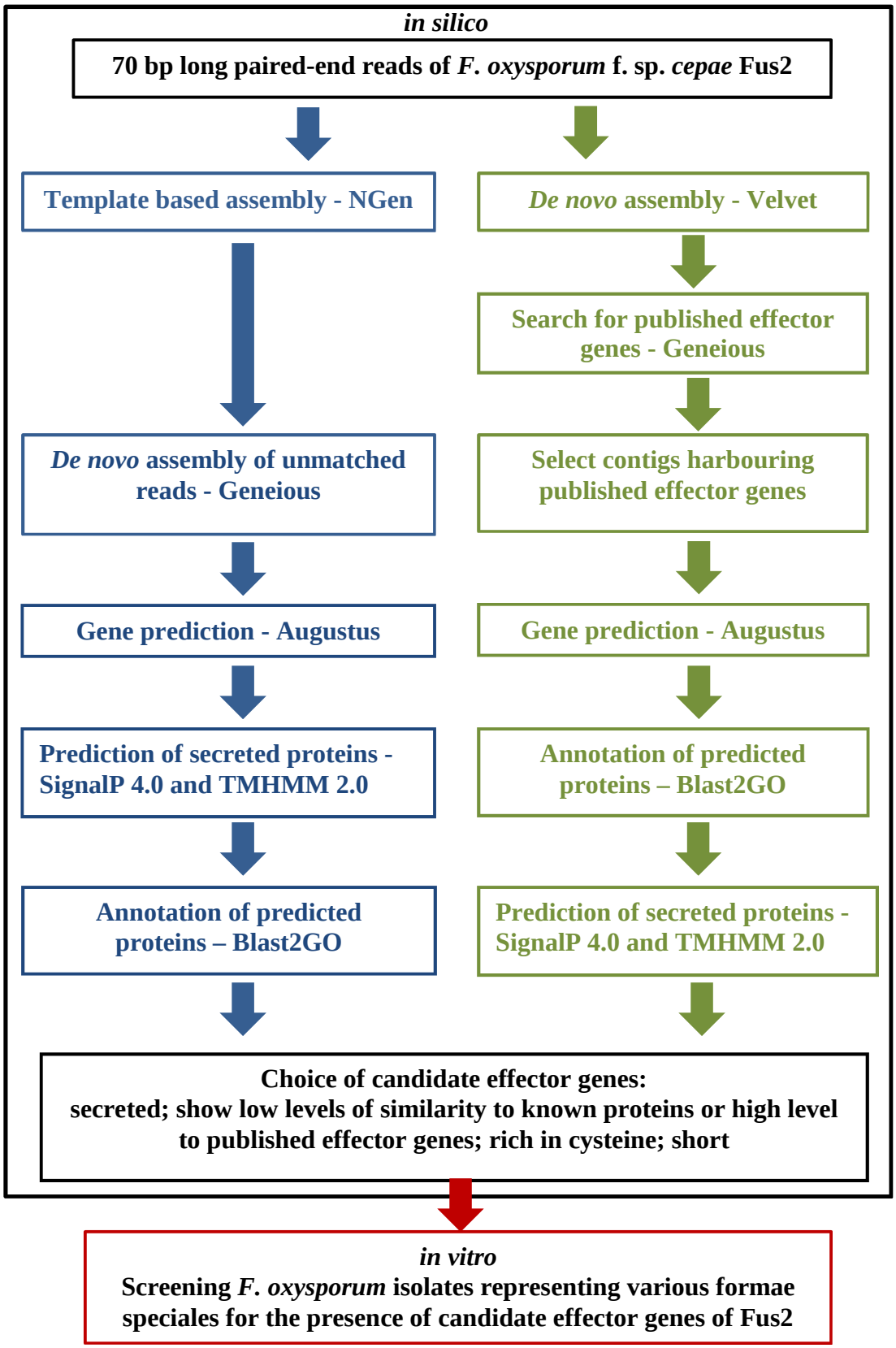


Figure 3. *In silico* analysis pipeline designed to identify putative effectors from the genome of *Fusarium oxysporum* f. sp. *cepae* (FOC) Fus2 followed by *in vitro* screening for the presence of these genes in representatives of various formae speciales.

4.3.3.2 Screening the Fus2 genome for the presence of published effector genes

The Fus2 genome was screened for the presence of effector genes (*SIX 1* to *SIX9* sequences were published by Lievens *et al*., 2009a and Thatcher *et al*., 2012) by using Custom Blast (Altschul *et al*., 1990), implemented in Geneious Pro (Drummond *et al*., 2011; Biomatters Limited).

4.3.3.3 Gene prediction on contigs

The web server Augustus (Augustus; Stanke and Morgenstrern, 2005) was employed to identify genes using default settings (predicting a few alternative transcripts on both strands) and *F. graminearum* as template.

4.3.3.4 Annotation of predicted genes

Predicted genes were functionally annotated by automated Blast-P and tBlastn-based annotation using Blast2GO (Conesa *et al*., 2005; Blast2GO). Signal peptides were predicted by SIGNALP 4.0 (Petersen *et al*., 2011; SignalP) by using the default settings. TMHMM v. 2.0 was used to filter out proteins containing transmembrane helices among secreted proteins (Krogh *et al*., 2001; TMHMM). The number of cysteines was counted by using a Python script (Holmes, 2012).

4.3.4 Choice of candidate effector genes for initial screening

Every secreted protein showing low similarity to previously published sequences was treated as a candidate effector protein. Due to the shortage of time, only eleven candidate effector genes were chosen for initial screening. Some other

criteria were also considered, for example: presence of N-terminal secretion peptide, similarity to other proteins (the lower similarity the better), presence of certain domains such as chitin or protein-binding domain, amino acid length (the shorter the better) and the number of cysteines (the more the better).

4.3.5 Screening of *F. oxysporum* isolates from diseased *Allium* crops and different formae speciales for the presence of known and candidate effector genes

Primer pairs were designed manually on the nucleotide sequences of ten candidate effector genes, *SIX3* and *SIX5* and tested on 25 of *F. oxysporum* isolates from diseased *Allium* crops and seven other formae speciales (Table 19; for primer sequences see Results). These isolates were chosen based on their aggressiveness in onion seedlings and represent the very aggressive (termed as FOC) and non-aggressive isolates with a few isolates from the middle of the scale (A13, 18B, 151, A28, Fus1 and 22).

Table 19. *Fusarium oxysporum* (25) isolates from diseased *Allium* crops and seven formae speciales used to screen for the presence of eleven candidate effector genes, *SIX3* and *SIX5*.

Culture	Host	formae speciales	Culture	Host	formae speciales
Fus2	onion	*cepae*	Fus1	onion	-
A14	onion	*cepae*	22	Welsh onion	*cepae*[b]
NL70_7	onion	*cepae*	Fom004	alfalfa	*medicaginis*[b]
SP7_2	onion	*cepae*	NRRL36425	tomato	*lycopersici*[b]
SM54	onion	*cepae*	PR5	leek	*cepae*[b]
D2	onion	*cepae*	SM105	onion	-
Fus3	onion	*cepae*	FOA5	asparagus	*asparagi*[b]
180	onion	*cepae*	NRRL26993	gladiolus	*gladioli*[b]
25	onion	*cepae*	NRRL26222	carnation	*dianthi*[b]
A13	onion	-	NRRL22538	onion	*cepae*[b]
FOA4	asparagus	*asparagi*	A28[a]	onion	-
PR7[a]	leek	*cepae*	18B[a]	leek	-
151[a]	onion	-			

[a] not used in screening for presence of ten candidate effector genes; [b] host specificity designated previously (Appendix I)
-: host specificity has not been defined

Composition of a 20 µl PCR mixture was: 1 µl DNA; 1 µl primer forward (20 µM), 1 µl primer reverse (20 µM), 10 µl RedTaq ready mix (Sigma-Aldrich) and 7 µl DNase free water (Sigma-Aldrich). Purification and sequencing of amplicons was carried out as described in Section 3.3.2.3. The PCR conditions were as follows: initial denaturation for 1.5 min at 94°C, followed by 35 cycles of 30 seconds denaturation at 94°C, annealing for 1.5 minute at the 57°C and extension for 2 minutes at 68°C. The program ended with a 10 minutes final extension at 72°C.

4.3.6 Screening of the Comparative *Fusarium* Database for the presence of predicted effector genes of Fus2

Genome sequences of ten additional *F. oxysporum* isolates representing several formae speciales became available in November 2011 from The Broad Institute, which allowed the screening of these isolates for the presence of candidate

effector genes selected primarily in this work. Predicted effector genes were manually annotated by BlastP and Blastn searches of the Comparative *Fusarium* Database (Broad Institute).

4.3.7 Evaluation and test bioinformatics tools and markers for the identification of further effector genes

A list of pathogenicity genes along with their amino acid sequences can be found on PHI-base (Baldwin *et al*., 2006; PHI-BASE). Fifteen genes have been published on PHI-base, which have been experimentally proven to be involved in pathogenicity of *F. oxysporum*. These genes were searched for by using Custom Blast in Geneious Pro (Drummond *et al*., 2011; Biomatters Limited). Novel motifs of secreted proteins were predicted by MEME v. 4.8.1 (Bailey *et al*., 2009; MEME). The NetNES 1.1 server was used to predict leucine-rich nuclear export signals (NES) in predicted secreted proteins (la Cour *et al*., 2004; NetNES). The Fus2 genome was screened for the presence of mimp1 elements by using custom blast in Genious Pro (Drummond *et al.,* 2011; Biomatters Limited). Fus2 contigs encoding *SIX* gene homologues were aligned to identify common motifs. Protein sequences of 49 secreted proteins of Fus2 were searched for the presence of published motifs such as RXLR-dEER (Whisson *et al*., 2007), LFLAK-DWL, (Birch *et al*., 2008), HaRxLs (Baxter *et al*., 2010), Y/F/WxC (Godfrey *et al.,* 2010), [L/I]xAR and [R/K]CxxCx12H (Yoshida *et al*., 2009), YxSL[R/K] (Levesque *et al*., 2010), G[I/F/Y][A/L/S/T]R (Catanzariti *et al*., 2006) and [R/K/H]x[L/M/V/F/Y/W]x (Kale, 2012) using a Python script (Holmes, 2012).

4.4 RESULTS

4.4.1 Preparation of DNA sample and genomic library for genome sequencing of FOC

A DNA concentration of 1730 ng/µl was obtained using the CTAB method, based on NanoDrop measurement. This sample was treated by RNase and purified by QIAEX kit (Qiagen) two consecutive times (sample A and B), which resulted in concentrations of 18.1 ng/ µl and 27.6 ng/ µl based on Qubit measurements (Table 20). There was a considerable difference in the concentrations measured by Nanodrop and Qubit. Surprisingly the purity of DNA extracts (A and B) were much lower compared to the original CTAB extracts (Table 20). *TEF* and ITS sequences did not indicate contamination or inhibition of the DNA sample (data not shown).

An assembly of 27,514,370 paired-end reads were generated which resulted 1,926,005,935 bp in total and provides about 40x coverage of the FOC genome.

Table 20. Quality and quantity of DNA sample used for genome sequencing of *Fusarium oxysporum* f. sp. *cepae* isolate Fus2.

Sample	Treatment	Concentration (ng/µl) by NanoDrop	Concentration (ng/µl) by Qubit	260/280 ratio*	260/230 ratio*
Fus2	CTAB extraction	1730	n.i.	2.04	2.10
Fus2 (A)	RNase treatment and desalting	89.7	18.1	1.31	0.31
Fus2 (B)	RNase treatment and desalting	94.4	27.6	1.30	0.33

Fus2 (A) and (B): A and B refers to that Fus2 CTAB extract was RNase treated and purified two (A & B) times; n.i.: no information
260/280 ratio: The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure for DNA.
260/230 ratio: The ratio of absorbance at 260 nm and 230 nm is a secondary assessment of the purity of DNA and RNA. Expected 260/230 values are in the range of 2.0-2.2.

4.4.2 *In silico* analysis pipeline based on *de novo* assembly to identify candidate effectors from the genome of Fus2

The assembly of 26,293,973 out of 27,514,370 70 base pair long paired-end reads by Velvet (Zerbino & Birney, 2008) resulted in 1511 contigs (maximum 1,268,561 bp long) and an N50 of 184 kb. The estimated size of Fus2 genome was 49.99 Mb. *In silico* screening of obtained contigs for the presence of nine published effector genes (*SIX1 – SIX9*) led to the discovery of the homologues of *SIX3*, *SIX5*, *SIX7* and *SIX9* in the genome of Fus2 (Table 21).

Table 21. Location and similarity of four published effectors of *Fusarium oxysporum* f. sp. *lycopersici* 4287 (FOL) genes in the genomes of FOL and *F. oxysporum* f. sp. *cepae* Fus2 (FOC).

Gene	Description	FOL	Similarity*	FOC
SIX7	Secreted in xylem 7	Chr 14, Sup 51,	87%	NODE_1912
SIX9	Secreted in xylem 9	Chr 14, Sup 22	82%	NODE_6613
SIX3	Secreted in xylem 3	Chr 14, Sup 36, 5017-5484+	86%	NODE_11583
SIX5	Secreted in xylem 5	Chr 14, Sup 36, 3273-3795-	73%	NODE_1095

*using tBLastx algorithm,
FOL: Chromosome of FOL4287; FOC: Contig of Fus2
Chr: chromosome, Sup: supercontig

Amino acid similarities between Foc-Six3, Foc-Six5, Foc-Six7 and Foc-Six9 and their homologs of FOL were 86, 73, 87 and 82%, respectively (Table 22). *Foc-SIX5* and *Foc-SIX7* were found on relatively short contigs: 1470 bp (NODE_1095) and 2798 bp long contigs (NODE_1912), respectively. *Foc-SIX9* and *Foc-SIX3* were encoded on slightly longer contigs: 6117 bp (NODE_6613) and 27198 bp (NODE_11583) (Table 22). *Foc-SIX3*, *Foc-SIX5*, *Foc-SIX7* and *Foc-SIX9* shared 90%, 87%, 90% and 88% nucleotide similarity with their homologues of FOL, respectively (Figure 29, Appendix VI). Forty-five mutations were present in *Foc-SIX3* when compared to *Fol-SIX3* and 23 of these mutations resulted in amino acid

change (Figure 29). Furthermore, it has to be noted that none of these homologues of FOC were found on the same contig, while only 1222 bp separates *Fol-SIX3* and *Fol-SIX5* in the genome of FOL (Table 21). Also, no homologues of *SIX3* (=*AVR2*) and *SIX5* of FOL, have been reported to be present from any other formae speciales previously; therefore they were subjected to further analysis.

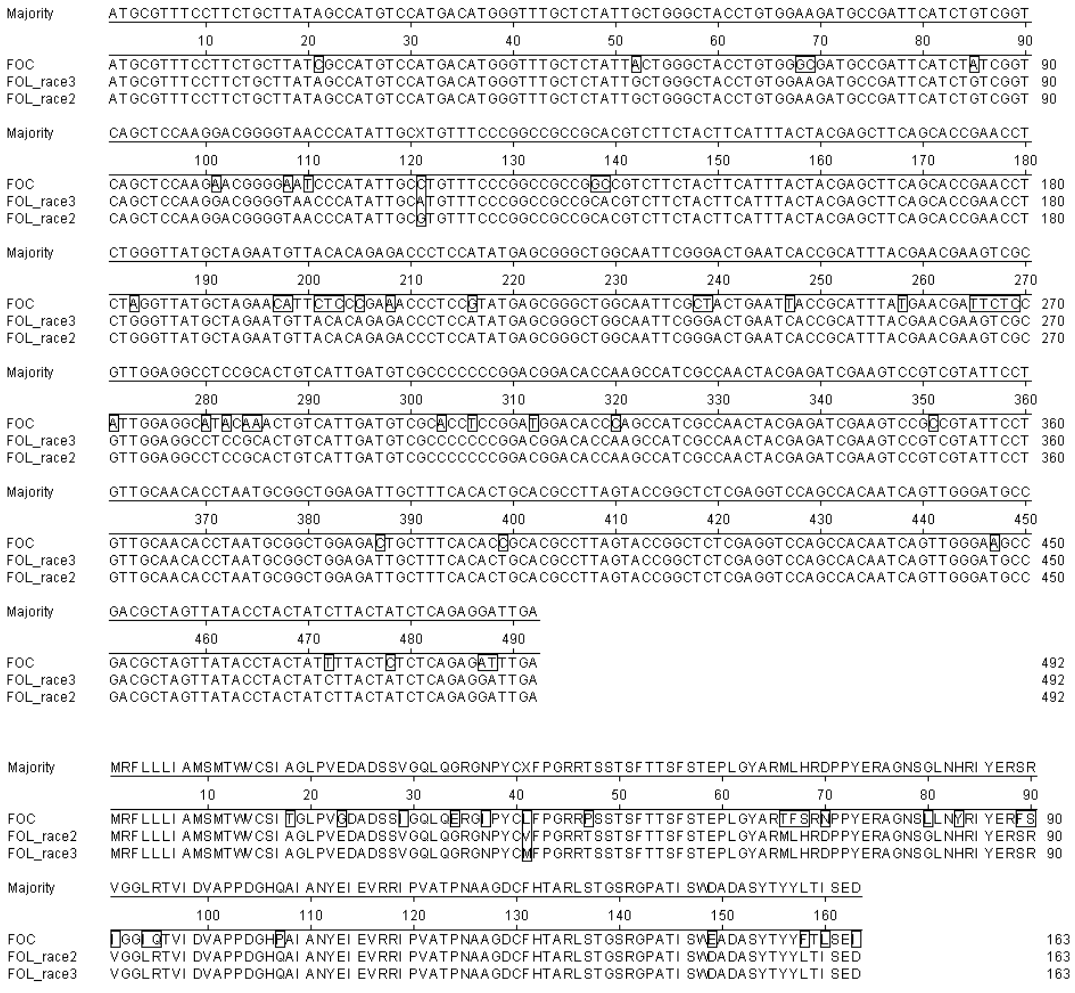


Figure 29. Multiple sequence alignment of *SIX3* nucleotide (top) and amino acid (bottom) sequences of *Fusarium oxysporum* f. sp. *cepae* (FOC) and *F. oxysporum* f. sp. *lycopersici* (FOL) race 2 and race 3.

A 27,198 bp long contig (NODE_11583) was subjected to gene prediction that resulted in nine predicted proteins (g1-g9) (Figure 30). The length of the

predicted proteins varied between 155 and 1154 amino acids (Table 22). Two out of nine predicted proteins (g3 and g5) did not show any similarities to known proteins deposited at NCBI database (NCBI) although based on coding sequence it showed 97% similarity to a hypothetical protein of the *Arabidopsis* pathogenic *F. oxysporum* isolate Fo5176. G8 was annotated as a homologue of *SIX3* by Blast2GO (Conesa *et al*., 2005). Annotation of the six remaining predicted proteins revealed the presence of homologues of two transposable elements (g6 and g7), a transcription factor (g4, btb poz domain-containing protein), a NADH oxidase (g1) and two hypothetical proteins (g2 and g9).

As *g5* did not show any similarity to any previously deposited sequences it was chosen for further analysis along with *SIX3* and *SIX5* effector genes. Overall, 25 *F. oxysporum* isolates representing seven formae speciales were screened, using newly developed primers (Table 23), for the presence of *SIX3, SIX5* and *g5* (Table 24). This experiment revealed that *SIX3* was only present in FOL as expected, a few isolates from onion and leek, and surprisingly, one isolate (NRRL 26222) representing *F. oxysporum* f. sp. *dianthi*. Interestingly, a homologue of *SIX3* was not detected in any isolates very aggressive toward onion seedlings.

Figure 30. Graphical presentation of the location, length and structure of predicted proteins on *SIX3* homologue coding contig, NODE_11538 of *Fusarium oxysporum* f. sp. *cepae* isolate Fus2.

Table 22. Amino acid length, percentage of similarity to closest hit, description of closes hit, results of annotation and similarity to genome sequences available on Broad Institute's website (BI%)* of 9 secreted proteins (g1-9g) coded on contig NODE_11538 of *Fusarium oxysporum* f. sp. *cepae* isolate Fus2.

Predicted gene	Sequence description	Length (aa)	Amino acid similarity (%)	Nucleotide similarity (%)	BI %
NODE_11583_**g1**	NADH oxidase	447	67	70	83
NODE_11583_**g2**.	hypothetical protein FOXB_04034 [*F. oxysporum* Fo5176]	190	57	78	94
NODE_11583_**g3**	secreted, hypothetical protein FOXB_04032 [*F. oxysporum* Fo5176]	319	n.i.	97	99
NODE_11583_**g4**	btb poz domain-containing protein	155	64	64	91
NODE_11583_**g5**	n.i.	244	n.i.	n.i.	n.i.
NODE_11583_**g6**	restless-like transposase	1154	69	69	98
NODE_11583_**g7**	Protein (transposase activity)	370	63	63	96
NODE_11583_**g8**	secreted in xylem 3	163	91	91	90
NODE_11583_**g9**	hypothetical protein FOXB_10733 [*F. oxysporum* Fo5176]	254	82	81	98

*using coding sequence; n.i. no information

Table 23. Sequences of three primer pairs designed based on the genome sequence of *Fusarium oxysporum* f. sp. *cepae* (FOC) Fus2 for the amplification and sequencing of three (candidate) effector genes, namely *SIX3*, *SIX5* and *g5*.

Primer	Sequence 5' → 3'	Primer	Sequence 5' → 3'
SIX5F	TGCGCTTCGAGTACATCTCT	g5f	GGAGGCATAGGTGACAGGAA
SIX5R	TGTTGAGTCTGCTCCTCCATT	g5r	GCATAAGGCCTCGATGTGAT
SIX3for	TCATCTRTCGGTCAGCTCCA	SIX3rev	ACTGATTGTGGCTGGACCTC

F, f, for: forward primer; r,R, rev: reverse primer
Target gene of primer is included in the name of the individual primers

Table 24. Overall 25 *Fusarium oxysporum* isolates from diseased *Allium* crops and six formae speciales screened for the presence of homologues *SIX3* and *SIX5* and candidate effector gene *g5*. Identical letters indicate identical sequences.

Culture	Host	Survival index[a]		*SIX7*[b]	*SIX3*	*SIX5*	*g5*
Fus2	onion	12.1		A	A	A	A
A14	onion	16.0		A	A	A	A
NL70_7	onion	16.		A	A	A	A
SP7_2	onion	16.8	Very aggressive[a]	-	-	-	-
SM54	onion	17.7		-	-	-	-
D2	onion	18.2		-	-	-	-
PR7	leek	19.1		-	-	B	-
Fus3	onion	19.7		A	A	A	A
180	onion	21.2		-	-	-	-
25	onion	22.4		A	A	A	A
A13	onion	29.0		a	A	-	A
18B	onion	30.5		-	-	A	C
Fus1	onion	32.3		-	-	-	-
151	onion	34.0		-	-	E	-
A28	onion	34.6		-	-	A	-
22	Welsh onion	36.1	Non-aggressive[a]	-	-	-	-
Fom004	alfalfa	38.0		-	-	-	B
NRRL36425	tomato	39.0		C	B	D	-
PR5	leek	39.5		-	-	A	-
SM105	onion	40.5		-	-	-	-
FOA4	asparagus	41.0		-	-	-	-
FOA5	asparagus	41.0		-	-	-	-
NRRL26993	gladiolus	41.7		-	-	-	-
NRRL26222	carnation	43.6		E	C	C	-
NRRL22538	onion	44.9		-	-	-	-

A-E: PCR product present, -: gene absent
[a]Survival index: Percentage survival of onion seedlings (angular transformed data), see Chapter 3
[b] For detailed description of *SIX7* screening see Chapter 3.

SIX3 sequences of all five isolates from onion and one from leek were identical (A), while isolates from tomato and carnation (*Dianthus* sp.) were representing two different sequence types (B and C) (Table 24). Overall, all isolates found positive for the presence of *SIX3* were also positive for *SIX7* (*SIX7* screening can be found in Chapter 3).

G5 was present (Table 24) in onion isolates that harboured *SIX3* and additionally in an isolate from leek (18B) and in *F. oxysporum* f. sp. *medicaginis* (isolate Fom004), although based on the sequences of both they were different (B and C) from onion isolates (A).

SIX5 was found in more isolates from *Allium* hosts compared to *SIX3*-positives: PR7, PR5, 151 and A28 were found to contain *SIX5* but not *SIX3* (Table 24). The only exception to this is one onion isolate, A13, which was *SIX3*-positive and *SIX5*-negative. Some of the non-aggressive isolates possessed identical *SIX5* sequence to very aggressive isolates. Overall, more sequence types (A - E) were distinguished within *SIX5* than *SIX3* and *SIX7*.

SUMMARY

Genome sequence analysis revealed that there are at least four homologues of published *SIX* genes (*SIX7, SIX3, SIX5* and *SIX9*) present in the genome of FOC Fus2. Additionally, a candidate effector gene (*g5*) was identified on the contig encoding *SIX3* of FOC. Novel primer pairs were developed to amplify three (candidate) effector genes of FOC, namely *SIX3, SIX5* and *g5*. Primers were screened on a set of 25 *F. oxysporum* isolates from diseased *Allium* species and different formae speciales. Interestingly, all isolates found positive for the presence of *SIX3* were also positive for *SIX7* (*SIX7* screening can be found in Chapter 3). The presence

/ absence of candidate effector genes of Fus2 suggests that the more of these genes that are present in a *F. oxysporum* strain the more likely it is to be more aggressive in onion seedlings. However, four of the very aggressive isolates (based on onion seedling assay), namely D2, 180, SM54 and SP7-2 were found negative for all three markers screened (*SIX3*, *SIX5* and *g5*).

4.4.3 *In silico* analysis pipeline based on template based assembly to identify candidate effector genes

The combination of template (FOL4287) based and *de novo* assembly of paired-end reads of Fus2 identified 85 unique contigs (10,162-87,720 bp long) which were not present in the genome of FOL4287 and presumably contain FOC-specific regions. In total, 509 proteins were predicted over these 85 contigs of Fus2 (Appendix VII).

In total 42 out of 509 proteins were predicted to encode N-terminal signal peptides. One protein (g66) out of 42 was predicted to be a transmembrane protein, therefore not listed in Table 25. Annotation of these 41 secreted proteins indicates that plant material degradation (29%), proteolysis (10%), chitin synthesis and metabolism (20%), cell defence (2%), protein-protein interaction (5%), hydrolysis (5%), pathogenicity (7%) seem to be important for host adaptation (Table 25, Figure 31). Unfortunately, 22% of predicted secreted proteins could not be annotated.

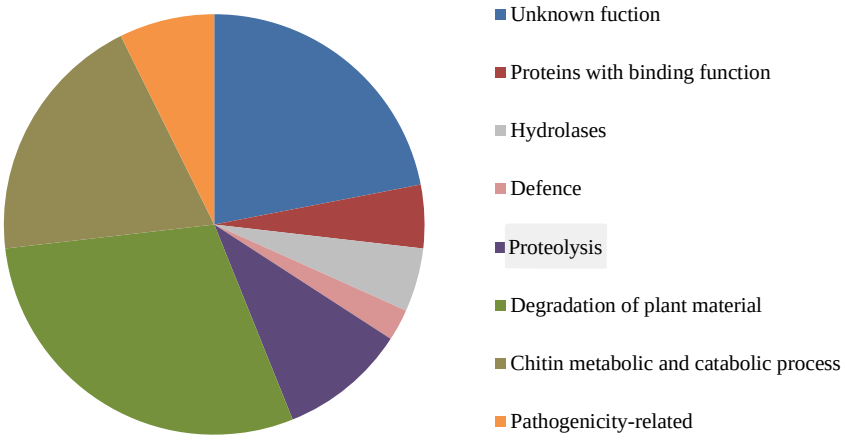


Figure 31. Functional annotation of secreted proteins of *Fusarium oxysporum* f. sp. *cepae* Fus2 not present in *F. oxysporum* f. sp. *lycopersici* 4287 genome.

Table 25. Nucleotide length, similarity to closest hit in NCBI database based on amino acid and nucleotide sequence and functional annotation of 41 secreted proteins coded on genomic regions of *Fusarium oxysporum* f. sp. *cepae* isolate Fus2 differing from the genome of *F. oxysporum* f. sp. *lycopersici* 4287 (Broad Institute). Proteins chosen for screening among *F. oxysporum* isolates from diseased *Allium* crops and different formae speciales are shown in **bold.**

Secreted protein	Annotation	Coding seq. (bp)	Amino acid %	Nucleotide %	No. of C
Contig_30_g239	hypothetical protein VDAG_09504	684	47	100	8
Contig_106_g471	**ankyrin repeat – protein binding**	**2364**	**47**	**91**	**20**
Contig_29_g210	metalloprotease mep1	447	50	100	3
Contig_106_g470	hypothetical protein FOXB_04997	2661	52	73	18
Contig_118_g463	**hypothetical protein VDAG_09127**	**1296**	**61**	**87**	**8**
Contig_7_g71	hypothetical protein FOXB_12412	1011	62	90	7
Contig_19_g156	amino acid oxidase	1080	64	91	8
Contig_39_g256	**para-nitrobenzyl esterase**	**1578**	**66**	**64**	**6**
Contig_75_g411	centromere microtubule binding protein cbf5	1227	66	94	10
Contig_23_g192	**class v chitinase**	**1871**	**67**	**88**	**37**
Contig_60_g342	**glucooligosaccharide oxydase**	**1494**	**67**	**46**	**2**
Contig_94_g480	**domain-cell wall catabolic process**	**1881**	**68**	**92**	**28**
Contig_60_g343	**amidohydrolase family protein**	**1509**	**70**	**56**	**1**
Contig_27_g203	para-nitrobenzyl esterase	1443	73	99	6
Contig_27_g209	alpha beta hydrolase	1023	73	96	1
Contig_93_g440	beta-galactosidase	2691	74	99	1
Contig_75_g413	exo-alpha-sialidase	1683	75	100	16
Contig_83_g416	beta-lactamase – antibiotic resistance	1260	76	100	1
Contig_94_g481	**chitinase**	**4509**	**76**	**85**	**39**
Contig_66_g374	**chitin deacetylase**	**1326**	**81**	**78**	**24**
Contig_23_191	**cell wall macromolecule catabolic process**	**5511**	**82**	**82**	**50**
Contig_8_g92	agglutinin-like protein als2 fragment	6522	87	86	23
Contig_2_g14	carbohydrate metabolic process	3015	90	97	12
Contig_8_g93.	class iii chitinase 1	927	90	92	6
Contig_92_g444.	hypothetical protein FOXB_13717	942	94	100	23
Contig_57_g401	hypothetical protein FOXB_02698	1185	95	100	10
Contig_17_g147	class 3 chitinase 2	978	96	93	7
Contig_57_g400	cell-cell adhesion	1737	96	79	0
Contig_67_g369	arylsulfatase-like protein – phenol metabolic process	1740	98	100	8
Contig_92_g443	glycoside hydrolase – cellulase activity	1116	98	99	5
Contig_5_g34	glycoside hydrolase family 115 protein	3192	99	84	3
Contig_24_g197	cysteine-rich repeat-metallopeptidase	1596	99	99	3
Contig_24_g199	alkaline proteinase (serine type)	1242	99	88	30
Contig_32_g247	alpha-glucanase	3399	99	95	37
Contig_34_g262	cas1 appressorium specific protein	906	99	100	4
Contig_40_g280	cell wall glycoprotein – carbohydate metabolism	897	99	99	18
Contig_67_g370	hypothetical protein FOXB_03179	1575	99	100	5
Contig_2_g8	small secreted protein	473	87	84	4
Contig_40_g277	lysophospholipase	1947	100	100	8
Contig_47_g333	hypothetical protein FOXB_03765	972	100	100	6
Contig_92_g442	pectin lyase	1134	100	95	7

Name of secreted protein contains information on the contig where it was predicted on, the individual name of the protein on given contig (number written after letter g)
Background colouring corresponding to Figure 31
No of C: number of cysteines in predicted protein

Several fungal chitinases were found among 41 secreted proteins of Fus2 (Table 25), such as V chitinase (bacterial/fungal) (g192) and class III (fungal/plant) chitinase 1 and 2 (g93 and g147). Fungal/bacterial chitinases are secreted enzymes that have been shown to play a role in the digestion and utilisation of fungal chitin, while fungal/plant chitinases have been shown to play a role during fungal morphogenesis (Hartl *et al*., 2012). Additionally, an unclassified chitinase (g481), a chitin deacetylase (g374), and a cell wall glycoprotein (g280) involved in carbohydrate metabolism were found. Two proteins, involved in fungal cell wall catabolic process, were also identified (g480 and g191). The position of these chitin catabolic proteins is really interesting as they are both located upstream to chitin metabolic proteins; g480 and g481 are encoded on contig 94 and g191 and g192 are located on contig 23.

A high proportion of predicted secreted proteins are potentially linked to the degradation of plant material (Table 25), for example g443 as cellulase, g442 as pectin lyase and enzymes involved in the modification of oligo- and monosaccharides (g342, g34, g247, g440, g14, g413, g256 and g203), phenol (g369) and lipid degradation (g277) were identified. Interestingly, cellulase g443 and pectin lyase g442 were found to be coded very closely on contig 92.

Three secreted proteins were predicted to be involved in adhesion (g400 and g92) and penetration (g262). G92 was similar to an agglutinin-like sequence (*Als2*) of *Candida albicans* that encodes a surface glycoprotein which is only expressed during infection (Hoyer *et al*., 1998) while the closest hit of g262 was Cas1, an appressorium specific protein that is expressed during appressoria formation of *Colletotrichum higginsianum* (Bakar *et al*., 2006). Two proteins were predicted to have hydrolase activity (g343 and g209), two proteins with protein-binding functions

(g471 and g411) in addition to three proteases (210, g197 and g199) and an amino acid oxidase (g156), and one protein (g416) was associated with antibiotic resistance that could be involved in plant material degradation and also in defence (Michielse *et al*., 2006, Rooney *et al*., 2005). A significant proportion (22%) of the proteins was identified as hypothetical proteins.

Ten predicted proteins were chosen for further analysis based on their nucleotide similarity to other proteins available on NCBI database (NCBI) and to genome sequences of formae speciales available on Broad Institute's database (Broad Institute) (Table 25). Nucleotide sequence similarity of chosen proteins varied between 47%- to 70%, while amino acid sequence similarity diverged between 47%- to 90%, predicted amino acid length was between 413-1818 amino acids and number of cysteines varied between 1- to 50 (Table 25). Five genes of Fus2 (*g481, g480, g191, g192, g374*) linked to chitin synthesis and degradation/modification were chosen for further analysis to be screened for their presence among 21 *F. oxysporum* isolates. *G48*0 and *g481* were only present in a few FOC isolates. In contrast, *g374, g191* and *g192* were present in the majority of FOC isolates (*g191* and *g192* was absent in isolate SM54) and some other formae speciales such as *medicaginis* and *asparagi*. Primer pairs were designed (Table 26) to amplify selected proteins from 21 *F. oxysporum* isolates representing six formae speciales and isolates with undefined host-specificity. Presence or absence of products was recorded and where it was possible isolates were divided into sequence type groups based on their sequence similarities (Table 27).

Table 26. Sequences of ten primer pairs designed based on the genome sequence of *Fusarium oxysporum* f. sp. *cepae* (FOC) Fus2 for the amplification and sequencing of ten candidate effector genes.

Primer	Sequence 5' → 3'	Primer	Sequence 5' → 3'
g343forward1	GCGGCACGATTATCACTTTT	g463forward1	GGCTGAAGTTATTGGCCTTG
g343reverse1	CAACCAAGAAAGTTGCGTGA	g463reverse1	GAGTGAAGCCTCTTCCGTTG
g342forward1	GCACATCCTTACGTGGCATC	g481forward1	CTTCTTTTCTTGCACCCTGTG
g342reverse1	TCACAACCTCCTCTGGCTCT	g481reverse1	GAAGGCGCTGTAAAGGTCAC
g256forward1	AGACCGCTAATTTGGCCTTT	g480forward1	TCCTCTGCTACTCGGTGGAC
g256reverse2	TGTAAAGTCGCCAACGTCAG	g480reverse1	GCGGTGATATTTGGGATGAC
g374forward1	AGCGTGCTGAGTGTGGTATG	g191forward1	GGGCCATCATCACTCTCATT
g374reverse2	CCGTACTGTGAGCAGCAGGT	g191reverse1	GCCGATCATCTCCGTATTGT
g471forward1	GGCTTATTGGCTTCTGTTGCC	g192forward1	ACCCAATTTCTCGCTGTCAC
g471reverse1	CCAGGCCGCATAGTGTTTAT	g192reverse1	GACCACCGCTTTGCTTTAAC

F, f, for: forward primer; r,R, rev: reverse primer
Target gene of primer is included in the name of the individual primers

Table 27. Presence/absence of ten candidate effector proteins (**in bold**) when screened on 21 *Fusarium oxysporum* isolates from diseased *Allium* crops and six formae speciales Sequence similarity is represented by letters (same letter refers to identical sequence type).

Isolate	Formae speciales	Survival index		Candidate effector genes									
				g 463	***g 374***	***g 256***	***g 471***	***g 191***	***g 192***	***g 342***	***g 343***	***g 480***	***g 481***
Fus2	*cepae*	12.1	Very aggressive[3]	A	A	A	A	A	A	A	A	A	A
A14	*cepae*	16.0		E	B	-	C	A	A	-	-	-	-
NL70_7	*cepae*	16.2		E	B	-	C	B	A	-	-	-	-
SP7_2	*cepae*	16.8		E	A	A	B	A	A	-	-	A	A
SM54	*cepae*	17.7		E	B	A	-	-	-	-	-	-	-
D2	*cepae*	18.2		E	A	A	B	A	A	-	-	-	-
Fus3	*cepae*	19.7		A	A	D	A	B	D	A	A	A	A
180	*cepae*	21.2		B	A	A	B	A	A	-	-	-	-
25	*cepae*	22.4		A	A	A	A	A	A	A	A	A	A
A13	ex. onion	29.0	Non-aggressive	-	-	C	A	A	A	A	A	-	-
Fus1	ex. onion	32.3		D	-	B	-	-	-	-	A	-	-
22	*cepae* [1]	36.1		C	-	-	-	-	-	-	-	-	-
Fom004	*medicaginis*	38.1		-	B	A	C	B	A	-	-	-	-
NRRL36425	*lycopersici*	39.0		-	B	A	-	A	A	-	-	-	-
PR5	ex. leek	39.5		-	B	A	C	D	C	A	A	-	-
SM105	ex. onion	40.5		-	C	E	-	-	-	-	-	-	-
FOA4	*asparagi*	41.0		G	A	A	C	A	E	A	A	-	-
FOA5	*asparagi*	41.0		C	-	A	-	+	-	-	-	-	-
NRRL26993	*gladioli*	41.7		-	-	+	-	-	-	-	-	-	-
NRRL26222	*dianthi*	43.6		F	-	-	-	-	-	-	-	-	-
NRRL22538	*cepae*[3]	44.9		-	-	A	-	C	B	-	A	-	-

[1] ex. Welsh onion- f. sp. designated by Dissanayake *et al* (2009ab), [2] ex. leek; [3] f. sp. designated by ARS
+: candidate effector present, but sequencing is still in progress; -: candidate effector does not present based on PCR reaction
[3]Survival index: percentage survival of onion seedlings (angular transformed), see Chapter 3

Two proteins showing similarity to glucooligosaccharide oxidase (*g342*) and para-nitrobenzyl esterase (*g256*) were chosen for further analysis based on their similarity to published sequences. *G342* was only found in some of the isolates, including representatives of *F. oxysporum* formae speciales *cepae*, *asparagi* and isolates non-aggressive in onion seedlings and it was identical in all isolates. Para-nitrobenzyl esterase-like gene (*g256*) was present in most of screened isolates (except A14, NL70_7, 22 and NRRL26222). Five *g256* sequence types were observed among 21 isolates, although A was the most common (Table 27).

Several of the screened candidate effector genes were limited to three or four isolates (Table 27). For example, isolate Fus2, Fus3 and 25 had all the candidate effector genes, and SP7-2 had all except two (*g342* and *g343*). Candidate effectors *g256, g374, g471, g191* and *g192* were present in highly aggressive isolates. Some candidate effectors seemed to show more variation than others: *g463* had seven sequence types (A – G), *g256* and *g192* comprised five (A – E), *g191* contained four (A – D), *g374* and *g471* three (A – C) and *g342, g343, g480* and *g481*comprised only one sequence type (A). In general, the higher number of candidate effectors detected in the genome of an isolate the more likely it was to be very aggressive in onion seedlings.

Sequences of the candidate effectors gene *g463* correlated with the results of the onion seedling assay. Sequence types A, B and E of *g463* could be promising to identify FOC as they were only present in very aggressive *F. oxysporum* isolates, while sequence types C, D, F and G were found in *F. oxysporum* isolates non-aggressive in onion seedlings (Table 27).

The genome sequences of ten additional *F. oxysporum* isolates became available in November 2011 from the Broad Institute, which allowed the screening of these isolates for the presence of candidate effector genes selected primarily in this work (Table 28). The only candidate effector which lacked any homologues was *g256,* while *g342, g343* and *g374* had one homologue each, *g480* and *g481* had two homologues each, *g463* and *g471* had seven homologues each, and *g193* had nine homologues.

Table 28. Presence / absence and nucleotide similarity (%) of candidate effector genes of Fus2 in the genome of ten *Fusarium oxysporum* isolates sequenced by The Broad Institute.

candidate effector	*pisi*	*radicis-lycopersici*	*conglutinans* race2	human	*lycopersici* race3	Biocontrol Fo47	*vasinfectum*	*raphani*	*cubense* race4	*melonis*	*lycopersici* race2 4287*	mitochondria*	Total
g256.	0	0	0	0	0	0	0	0	0	0	0	0	0
g342	0	0	92	0	0	0	0	0	0	0	0	0	1
g343	0	0	95	0	0	0	0	0	0	0	0	0	1
g374	0	99	0	0	0	0	97	0	0	0	0	0	1
g480	98	0	0	0	0	0	0	97	0	0	0	0	2
g481	96	0	0	0	0	0	0	96	0	0	0	0	2
g463	88	99	0	0	0	0	0	0	0	0	0	0	2
g192	82	99	81	82	98	99	0	81	0	0	0	0	7
g471	0	97	0	87	82	97	82	81	93	0	0	0	7
g191	99	93	98	85	93	96	98	82	95	0	0	0	9
Total	4	4	4	3	3	3	2	2	2	0	0	0	

Hosts of isolates: *Pisum* (*pisi*), *Brassica/Arabidopsis* (*conglutinans* race2), *Solanum lycopersicum* (*radicis-lycopersici*, *lycopersici* race2 and race3), *Homo sapiens* (human), *Gossypium* (*vasinfectum*), *Raphanus/Arabidopsis* (*raphani*), *Musa* (*cubense* race 4), *Cucurbita* (*melonis*)
**lycopersici* race 2 4287 and mitochondria were used for the original template based assembly

None of the candidate effectors had a hit against *melonis*, *lycopersici* race 2 and mitochondrial genomes (Table 28), which suggests that the template based assembly was applicable to identify all homologues. Moreover, it reveals that lineage specific regions are present in *pisi* (four hits), *radicis-lycopersici* (four hits), *conglutinans* race2 (three hits), *lycopersici* race3 (three hits), *vasinfectum* (two hits), *raphani* (two hits), *cubense* race 4 (two hits), human pathogenic (three hits) and even in the biocontrol strain Fo47 (three hits).

SUMMARY

Genome sequence analysis revealed that there are at least 509 genes present in the genome of FOC Fus2 which are not present in the genome of tomato pathogen FOL 4287, such as pisatin demethylase. In total, 40 proteins of Fus2 were predicted to possess N-terminal signal peptide. These secreted proteins are predicted mainly to

be involved in the degradation of plant material (29%), chitin synthesis/metabolism (20%) or have unknown function (22%). In total eleven candidate effector genes of FOC were tested on a set of 20 *F. oxysporum* isolates from diseased *Allium* species and different formae speciales. The presence / absence of eleven candidate effector genes of Fus2 suggests that the more of these genes that are present in a *F. oxysporum* strain the more likely it is to be more aggressive in onion seedlings. One candidate effector gene with unknown function of Fus2 was found promising for the specific identification of FOC isolates based on this preliminary data. This gene was *g463.*

4.4.4 Annotation of genes of Fus2 without N-terminal secretion signal peptide

Proteins were annotated using Blast2GO (Conesa *et al.*, 2005) and about 25% of these predicted proteins could not be functionally classified on the basis of homology to known proteins (Appendix VI). The rest of the genes were involved in the degradation of plant cell wall, translocation of metabolites, synthesis of enzymes, polyketides, small signalling proteins, transcription factors, proteins involved in fungal growth and pathogenicity. There are a number of proteins predicted to have role in fungal pathogenesis, such as pisatin demethylase enabling the infection of pea by degrading phytoalexin pisatin (Temporini and VanEtten, 2004).

One of the most striking observations is the discovery of enzymes involved in the synthesis of various toxins in the genome of Fus2, including mosquitocidal toxin, aflatoxin and trichothecene. Mosquitocidal toxins are pore-forming protein toxins and were described originally from *Bacillus sphaericus* as insecticides (Maagd *et al*., 2003). A ketoreductase, nor-1, was identified, which is involved in aflatoxin

synthesis in *Aspergillus* species (Zhou and Linz, 1999). Trichotechene production requires the presence of several genes, however only trichothecene 3-o-acetyltransferase and tri15 were identified.

The closest homologue of some of the predicted proteins generally occurs in plants and animals. A protein which is normally found in plants was gibberellin-3β-hydroxylase, involved in the synthesis of plant hormone gibberellin (Cadman *et al*., 2006). A glycoprotein similar to von Willebrand factor, which is involved in adhesion to wound sites in mammals (Ruggeri and Ware, 1993), was also predicted.

A homologue of a reverse transcriptase was also found; active reverse trascriptases were found on two linear mitochondrial chromosomes or mitochondrial retroplasmids of *F. oxysporum* (Kistler *et al*., 1997, Simpson *et al.,* 2004). Two proteins involved in vegetative compatibility were also predicted in Fus2. Interestingly, two very common vegetative compatibility groups (VCGs) and several less frequent VCGs have been found within FOC (Southwood *et al.,* 2012).

4.4.5 Evaluation and test bioinformatics tools and markers for the identification of further effector genes

In the genome of Fus2 by an *in silico* screening using contigs published in PHI-BASE database of 15 pathogenicity genes were identified (Table 27) (Winnenburg *et al*., 2008).

All 15 of these genes were coded on the “core” genome (core genome defined by Ma *et al*, 2010) of FOL4287 unlike the four *SIX* genes coded on the “lineage specific” region (Table 29). Eleven pathogenicity genes of FOL4287 showed a high level of amino acid similarity (<98%) to the homologues of Fus2. In contrast, *ARG1*,

FMK1, FOW1, and *FOXG_00016* shared 87, 92, 89 and 87% amino acid similarities, respectively.

Table 29. Location and similarity of 15 pathogenicity genes in the genomes of *Fusarium oxysporum* f. sp. *lycopersici* 4287 (FOL) and *F. oxysporum* f. sp. *cepae* Fus2 (FOC).

Gene	Description	FOL4287	Similarity*	FOC Fus2
ARG1	argininosuccinate lyase	Chr 5	87%	NODE_1080
FMK1	mitogen-activated protein kinase	Chr 4	92%	NODE_6668
FGA1	guanine nucleotide-binding protein alpha subunit	Chr 4	100%	NODE_922
FOW1	putative mitochondrial carrier protein	Chr1	89%	NODE_2884
CHSV	class V chitin synthase	Chr 4	100%	NODE_2775
FGB1	G protein beta subunit	Chr 10	100%	NODE_3106
foSNF1	protein kinase	Chr 7	99%	NODE_10874
FOXG_00016	unknown	Chr 1	87%	NODE_2669
PacC	pH transcription factor	Chr 5	100%	NODE_7884
CHS2	chitin synthase class II	Chr 7	98%	NODE_4211
CHS7	chitin synthase chaperone-like protein	Chr 7	100%	NODE_11735
FGA2	G-protein alpha subunit	Chr 2a	99%	NODE_1531
FRP1	F-box protein	Chr 1	100%	NODE_2669
GAS1	beta-1,3-glucanosyltransferase	Chr 4	100%	NODE_2377
FOW2	Zn(II)2Cys6-type transcription regulator	Chr 2a	100%	NODE_7884

*using tBLastx algorithm, FOL: Chromosome of FOL4287, FOC: Contig of Fus2

Contigs harbouring *SIX3, SIX5* and *SIX7* were screened for mimp elements in their promoter region to test whether it can be useful marker for the identification of additional *SIX* genes. This screening revealed the presence of a mimp element in the promoter of *SIX7*, but not *SIX3* or *SIX5*, although the contig where *SIX5* was found is very short and may not cover the entire promoter region. This indicated that the presence of mimp elements in the promoter region of candidate genes could be a

useful marker for the identification of some *SIX* genes in the genome of Fus2. All 1511 contigs of Fus2 were screened for the presence of mimp elements which identified 61 mimps in this genome. These 61 contigs can be subjected to annotation and based on the location of mimp elements additional *SIX* genes could be predicted.

SIX3, *SIX5* and *SIX7* harbouring contigs were compared which led to the identification of a short (34bp) identical sequence downstream (3' prime end) of the coding regions of *SIX5* (NODE_1095) and *SIX3* (NODE_11538). A 19 bp long region (CCGCGTTGAGGTTTTCCCA**)** of this sequence showed 100% similarity a crinkler (CRN) protein (GENE ID: 9477723 PITG_00601) of *Phytophthora infestans*.

A novel motif L[HF][HS][HS]A was found among the secreted proteins by MEME v. 4.8.1 (Baily *et al*., 2009). This motif was present in four proteins out of 41, namely g484, g14, g473 and g346. Leucine-rich nuclear export signals (NES) were predicted by the NetNES 1.1 server among secreted proteins (la Cour *et al*., 2004) (Table 30).

Table 30. Leucine-rich nuclear export signal containing secreted proteins of Fus2 and amino acid sequences of corresponding signals.

Predicted secreted protein	Leucine-rich nuclear export signal
Contig_92_g444	LLDGHLPV
Contig_66_g374	LAADKLLSPL
Contig_47_g333	VGKDLEALEL
Contig_40_g280	LAGAIGAAAVAL

Forty-one secreted proteins of FOC not present in the genome of FOL were screened for the presence of published motifs (Appendix VIII). RXLR motifs were found in four proteins (g343, g413, g463 and g470), dEER in five proteins (g191,

g192, g256, g416, g470), Y/F/WxC in 14 proteins (g14, g71, g191, g192, g197, g247, g262, g277, g333, g374, g401, g470, g480, g481), HaRxLs in three proteins (g199, g203, g416), DWL in three proteins (g411, g443 and g470), [L/I]xAR in three proteins (g191, g369 and g470). [R/K/H]x[L/M/V/F/Y/W]x was present in all proteins except three (g262, g280 and g51). In contrast LFLAK, [R/K]CxxCx12H, G[I/F/Y][A/L/S/T]R and YxSL[R/K] were not found in any of the proteins.

SUMMARY

The use of bioinformatics tools/database/markers was explored for the *in situ* identification of (candidate) effector genes in the genome of FOC.

PHI-BASE (Winnenburg *et al*., 2008), a database of previously published pathogenicity genes was searched for genes identified in *F. oxysporum* formae speciales *lycopersici* and *pisi*. In total, 15 genes were identified and were exploited to further analysis. Among these, homologues of four (*ARG1*, *FMK1*, *FOW1*, and *FOXG_00016*) were showing lower (87-92%) sequence similarity, therefore could be useful to further investigate their use in FOC-specific molecular test).

Previously, it was shown that mimp elements in the promoter region of candidate genes could be a useful marker for the identification of *SIX* genes (Schmidt, 2012). Mimp element was identified in the promoter of *SIX7* of FOC, but not *SIX3,* therefore this marker is only useful for the identification of some candidate effector genes in the genome of FOC. Additionally, multiple sequence alignment of *SIX3, SIX5* and *SIX7* harbouring contigs of FOC led to the identification of an identical (34bp) sequence upstream the coding region, which showed also seems to present in a Crinkler protein of *P. infestans*.

Four secreted proteins were predicted to comprise leucine-rich nuclear export signals (NES) by the NetNES (la Cour *et al*., 2004). A novel motif was found among the secreted proteins by MEME v. 4.8.1 (Baily *et al*., 2009). Ten previously published motifs present in various effectors were identified by a Python script (Holmes, 2012) among secreted proteins.

4.5 DISCUSSION

4.5.1 Preparation of DNA sample and genomic library for genome sequencing of FOC

To date twelve genome sequences of *F. oxysporum* have been published (Broad Institute, Thatcher *et al.,* 2012). The genome sequence of Fus 2 provides a novel resource to enhance our understanding of the *F. oxysporum* – non-model plant interaction. Previously published plant pathogenic *F. oxysporum* strains typically cause wilt disease, therefore the sequence of a root and bulb rot causing strain, FOC, could be useful to have a better understanding of formae speciales causing these symptoms. Some rot causing *F. oxysporum* formae speciales colonise only the cortex, some both cortex and xylem (Baayen and Rijkenberg, 1999, Baayen *et al.*, 2000).

Genome sequencing requires good quality (i.e. unsheared) DNA extract to obtain large insert libraries. Therefore CTAB method was chosen to extract a large amount of DNA of FOC strain Fus2. This primary extract contained high amounts of fairly pure DNA, therefore it was considered as a suitable method for the preparation of genomic libraries. This primary extract was RNase treated and further purified by QIAEX kit (Qiagen). This kit binds DNA to beads instead of filters during the purification process, therefore in theory these results in a least sheared DNA sample. Unfortunately, this kit gave at least x20 diluted DNA sample by the end of the purification compared to the CTAB extract. A recent protocol by the 1000 Fungal Genomes Project suggested the use of a modified version the protocol of Fulton *et al.* (1995) with the combination of Qiagen genomic tip 500/G which would have been a solution for the clean-up phase (1000 Fungal Genomes Project). Some publications

reported that commercially available genomic DNA extraction kit gave DNA applicable for genome sequencing (Tom Hsiang, University of Guelph, pers. comm.).

4.5.2 *In silico* analysis pipeline based on *de novo* assembly to identify putative effectors from the genome of Fus2

The estimated size of Fus2 genome was 49.99 Mb which is smaller compared to the 61.36 Mb genome of FOL4287, but on average (46.55 Mb – 55.18 Mb) compared to the ten other *F. oxysporum* genomes sequenced by the Broad Institute.

The first effector gene, *SIX1*, of FOL was cloned in 2004 by Rep and co-workers (Rep *et al*., 2004) and to date in total 15 *SIX* genes have been discovered in the genome of FOL (Takken and Rep, 2010; Sarah Schmidt, University of Amsterdam, pers. comm.). Only the roles of *SIX1* (=*AVR3*), *SIX4* (=*AVR1*) and *SIX3* (=*AVR2*) have been confirmed (Rep *et al*., 2004; Houterman *et al*., 2008; Houterman *et al*., 2009). Homologues of *SIX6*, *SIX7*, *SIX8*, *SIX9*, *SIX1* (=*AVR3*) and *SIX4* (=*AVR1*) has been reported from *F. oxysporum* ff. spp. *lilii*, *melonis*, *radicis-cucumerinum*, *vasinfectum* and *Arabidopsis*-infecting strain Fo5176, respectively. *In silico* screening of the Fus2 genome led to the discovery of the homologues of *Foc-SIX3*, *Foc-SIX5*, *Foc-SIX7* and *Foc-SIX9*. This is the first report of the discovery of a homologue of *Fol-AVR2* (=*Fol-SIX3*) and *Fol-SIX5* in FOC, another f. sp. than FOL. The *SIX3* gene was thought to be unique to FOL (Takken and Rep, 2010). The *SIX3* homologue shows considerable (86%) amino acid differences. This suggests that the *SIX3* gene of these two formae speciales was not recently gained. For example, the homologue of *SIX4* (=*AVR1*) was found in an *Arabidopsis*-infecting strain Fo5176 which differed in only two amino acids when compared to the *SIX4* of FOL (Thatcher *et al*. 2012). The high level of conservation was proposed to be caused by

recent gain of this gene from a common ancestor. The other possibility is that the *SIX3* gene of FOC is under strong selection pressure to adapt to its host. The presence of homologues *SIX3* in the genome of FOC might indicate the presence of the corresponding resistance gene (*I-2*) of tomato in the onion genome. As no onion genome sequence available at the moment it could be investigated by hybridisation and by designing degenerate primers on resistance gene *I-2* of tomato.

Nine genes were predicted on the contig containing *SIX3* which showed similarity to hypothetical proteins and active transposable elements. This agrees with previous findings; lineage specific regions of FOL4287 were rich in transposable elements and most of the predicted proteins were uncharacterised (Ma *et al*., 2010). Strikingly, *Fol-SIX3* and *Fol-SIX5* are coded in close proximity, but not their homologues in the genome FOC Fus2. This rearrangement is very likely to be the result of transposon activity. For example, a novel biotype of FOL race 3 isolate was found that had *AVR1*, but it was gene truncated by the transposon, mobile genetic elements were proposed to be the driving forces underlying race evolution (Inami *et al.*, 2012). Three active transposable elements were predicted on the contig coding *SIX3* of FOC. It was impossible to functionally characterise a non-secreted short protein (g5), which is a characteristic of *SIX* genes (Rep *et al*., 2004; Houterman *et al*., 2008).

Screening of 75 FOL isolates representing all three races for the presence of *SIX1-SIX7* genes revealed that *SIX1-SIX3* and *SIX5-SIX7* were present in all FOL isolates, while *SIX4* was present only in FOL race 1 (Lievens *et al*., 2009a). Moreover, sequences of individual *SIX* genes were identical for all FOL isolates. In contrast, *SIX3, SIX5 and g5* were not found in all *F. oxysporum* isolates very aggressive in onion seedlings, although these genes were found conserved in those

aggressive isolates tested positive for their presence. This high level of conservation suggests that these genes have an evolutionary conserved function that constrains gene diversification. The presence of identical sequence type of *SIX5* in some of the isolates tested as weakly and very aggressive in onion seedlings could suggest that these isolates have similar host range or way of infection.

Overall, *SIX3*, *SIX5* and *g5* are useful for the robust identification of some of the very aggressive FOC isolates, although some FOC isolates seem to lack these genes therefore further markers are needed to identify the "*SIX*-negative" FOC isolates. However, it is also possible that some of the *SIX* genes are present in these isolates, but did not amplify due to variation in *SIX* gene regions targeted by primer used. Genome sequencing of these isolates or hybridisation could help to clarify this. It is still useful information that homologues of *SIX3* (=*AVR2*) and *SIX5* are present in both FOL and FOC.

Expression analysis of these genes *in planta* is also needed and phenotypic analysis of deletion and overexpressing mutants to clarify the role of these genes in FOC. In case Foc-*SIX* genes are functioning and differentially expressed *in planta* that could suggest that FOL and FOC are using similar mechanism to manipulate host cells.

4.5.3 *In silico* analysis pipeline based on template based assembly to identify candidate effectors in the genome of Fus2

Genome sequence based assembly identified reads which showed high similarity to the template (FOL4287) and left aside the unmatched reads. In theory, the unmatched reads correspond to unique genomic regions, which do not occur in the "core *Fusarium* genome" (regions present in *F. verticillioides, F. graminearum* and *F. solani*) or in the *lycopersici*-specific lineage specific regions (Ma *et al*., 2010). The unmatched (presumably including FOC-specific) reads were assembled using *de novo* assembly which resulted in 85 contigs. This approach hugely narrowed down the amount of data to be analysed further not to mention the time and computational power required. This approach could be further developed by using other *F. oxysporum* genome sequences (being) published as templates to identify unique regions of Fus2.

In total, 509 proteins were predicted over the 85 contigs of Fus2 which do not show similarity to those encoded in the genome of FOL4287. In total 17,708 genes were predicted from the genome of FOL4287 excluding the mitochondrial genome (Broad Institute). This again, shows that the combination of template based and *de novo* assembly hugely reduced the number of genes to be analysed. These 509 genes could be the key to finding what makes a *F. oxysporum* isolate aggressive in onion seedlings rather than tomato. These proteins could be interacting with the plant directly or indirectly in the same time (Aslam *et al*., 2009). It is also possible that some of these genes are pseudogenes (Campbell *et al*., 2012). The accuracy of gene prediction could be improved by using the combination of several gene prediction software packages such as FGENESH (Softberry) and / or by "training" these prediction programs by using *F. oxysporum* EST data (Stanke *et al*., 2008).

In total, 42 out of 509 proteins were predicted to encode N-terminal signal peptides and only one of them was predicted to be a transmembrane protein. These results show that the latest version of SIGNALP program provides a fairly good prediction, but the employment of TMHMM is very valuable to improve prediction of secreted proteins. It also has to be considered that there are independent ways of protein transportation which do not involve the presence of N-terminal signal peptides (Ridout *et al*., 2006). For example, the RXLR-dEER motif of *Phytophthora* fused with GFP protein can be transported into plant cells (Dou *et al*., 2008). Moreover, there are *in silico* tools already available to predict "non-classical" secreted proteins of bacteria and mammals (Bendtsen *et al.,* 2005).

In total, 41 secreted proteins were predicted over these 85 contigs of Fus2. These are predicted to be involved in the degradation of plant material, proteolysis, chitin synthesis/metabolism and defence, pathogenicity or have unknown function. The majority (29%) of the 41 secreted proteins were annotated to be involved in the degradation of plant material (cellulose, pectin, oligo- and polysaccharides). One protein was found to be involved in the degradation of phenolic compounds, which could indicate its role in lignin (phenolic polymer) or toxic phenolic compound degradation (Michielse *et al*., 2009). Only 20 % of the genes encoded on the lineage specific region of FOL4287 could be functionally classified, but some of the annotated genes were secreted enzymes predicted to degrade or modify plant or fungal cell walls (Ma *et al*., 2010). Pectic enzymes, exo-polygalacturonase and endopectin-trans-eliminase of FOC, were observed in the onion stem at the beginning of colonization (Holz and Knox-Davies, 1985). It is possible that some of these proteins also assist the saprophytic lifestyle to utilize decaying plant material (Kim *et al*. 2007).

Two proteins showing similarity to glucooligosaccharide oxidase (g342) and para-nitrobenzyl esterase (g256) were chosen for further analysis based on their similarity to published sequences. G342 was only found in a few isolates and it was identical in all isolates, which either means that this gene was gained recently from a common origin or it has a conserved function. In contrast, para-nitrobenzyl esterase-like protein (g256) was present in most of screened isolates and showed sequence variation. In general, none of these genes aligned with aggressiveness in onion seedlings.

Relatively large proportions (20%) of secreted proteins were predicted to be involved in chitin synthesis or degradation. Chitin is one of the most important fungal pathogen-associated molecular patterns (PAMPs) which can be recognized by plants and mammals and can trigger the immune response (Vega and Kalkum, 2011). In rice, the chitin elicitor binding protein (CEBiP) recognizes chitin oligosaccharides released from the cell walls of *M. oryzae,* which overcomes PAMP-induced immunity by secreting an effector protein, secreted LysM Protein1 (Slp1) (Mentlak *et al*., 2012).

Chitin synthesis has been shown to be crucial for pathogenicity; several chitin synthase gene knock-out mutants lost virulence or had reduced virulence (Martinez-Rocha *et al*., 2008; Martin-Urdiroz *et al*., 2008). Proteins involved in chitin synthesis of FOC are predicted to be secreted, which disagrees with previous findings; known chitin synthases are not integral membrane-bound proteins (Roncero *et al*., 2002). The role of chitinases in terms of virulence has not been proved in *F. oxysporum*, although they were detected by insertional mutagenesis as potential pathogenicity related genes (Michielse *et al*., 2009). Chitinase mutants of *Aspergillus fumigatus* showed only a limited reduction in the total chitinolytic activity and had no growth

or germination defects (Alcazar-Fuoli *et al*., 2011). The presence of genes linked to chitin synthesis and their utilization is crucial in the process of cell wall remodelling to respond to host defence (Michielse *et al*., 2009). Also, some of the secreted enzymes modify pathogen cell walls, e.g. chitin deacetylase, in order to protect themselves from enzymatic hydrolysis (El Gueddari *et al*., 2002). An avirulence protein of *C. fulvum,* Avr4, contains a chitin-binding motif which protects the fungal cell wall from plant chitinases (van den Burg *et al*., 2006). Interestingly, some of the chitin catabolic genes (*g191* and *g480*) of Fus2 were encoded upstream of chitin metabolic genes (*g192* and *g481*), suggesting that their regulation is linked, which would allow for the continuous remodelling of fungal cell walls. Four genes (*g481, g480, g191, g192*) linked to chitin synthesis and degradation along with chitin-deacetylase (*g374*) of Fus2 were chosen for further analysis. *G480* and *g481* were only present in a few FOC isolates suggesting that they might be "accessory" genes of FOC isolates. In contrast, g191 and g192 were present in the majority of FOC isolates (except SM54) and some other formae speciales such as *medicaginis* and *asparagi*. Wheat pathogen *Mycosphaerella graminicola* has eight small accessory chromosomes which have a role in host adaptation (Stukenbrock *et al*., 2010). G374 was present in all FOC isolates and in a few additional isolates, which suggest that chitin-deacetylase is essential for pathogenicity in onion. Unfortunately, none of the sequence types of *g481, g480, g191, g192* and *g374* aligned with pathogenicity.

Two proteins were predicted to have hydrolase activity (g343 and g209). Hydrolases were predicted from *F. graminearum* secretome (Brown *et al*., 2012; these proteins could be involved in the degradation / modification of plant or fungal material and tissue maceration. Amidohydrolase *g343* was chosen for further analysis based its low sequence similarity to known proteins. It was detected in

several highly and non-aggressive *F. oxysporum* strains and its sequence was highly conserved, which suggests that this protein has a recent common origin. Unfortunately the presence of *g343* did not correlate with pathogenicity.

Two proteins were predicted to have protein-binding function (g471 and g411) additionally to three proteases (g210, g197 and g199) and an amino acid oxidase (g156) that could be involved in plant material degradation and also in defence (van der Hooven *et al*., 2001; Michielse *et al*., 2009). Genes linked to protein degradation, translocation and amino acid metabolism were found to be important in pathogenesis of *F. oxysporum* (Michielse *et al*., 2009). Also, proteinases are commonly found to be involved in cell defence. For example, Avr-Pita effectors from *M. oryzae* show homology to zinc-dependent metalloproteases (Jia *et al.,* 2003). A metalloproteinase (mep1) was described from the human pathogen *Coccidioides posadasii* that digests an immunodominant cell surface antigen and prevents host recognition of endospores (Hung *et al*., 2005). A secreted metallopeptidase was found to be expressed exclusively during the infection of wheat by *F. graminearum* (Paper *et al*., 2007). An entire class of Kazal-like extracellular serine protease inhibitors directly interacting with and inhibiting host proteases was described from *P. infestans* (Tian *et al*., 2005). AvrP123 of *M. lini* contains a Kazal serine protease inhibitor signature which suggests that it has a role in the inhibition of host proteases (Catanzariti *et al*., 2006). *G471* was chosen for further analysis as its presence of an ankyrin repeat suggests that it is involved in protein-protein interaction. This protein was found in almost all very aggressive isolates and a few non-aggressive isolates including *F. oxysporum* ff. spp. *medicaginis* and *asparagi*. Three sequence types were observed but there was no relationship between aggressivess in onion seedlings.

Three proteins were predicted to be involved in adhesion (g400 and g92) and penetration (g262) based on sequence similarity. In an isolate of *F. oxysporum* which infects tomato, localized hyphal swellings were observed which may represent appressorium-like penetration structures (Czymmek *et al*., 2007). A set of *F. oxysporum* isolates will be screened for the presence of these proteins in order to establish a better understanding of their pathogenicity and to investigate whether or not they differ between isolates.

A high proportion (22%) of the proteins was identified as hypothetical proteins, which agrees with previous findings. Half of the *F. graminearum* secretome encoded for proteins of unknown function (Brown *et al*., 2012). Moreover, 80% of the genes encoded on the lineage specific regions of FOL4287 could not be functionally classified (Ma *et al*., 2010). A conserved hypothetical protein (FOXG_09487) was verified by reverse genetic approaches and shown to be important for *F. oxysporum* pathogenicity (Michielse *et al*., 2009). One candidate effector gene, *g463*, without functional classification of Fus2 was chosen for further analysis. Preliminary data based on the screening of 21 *F. oxysporum* isolates representing various formae speciales suggests that some of the sequence types (A, B and E) could be linked to pathogenicity. The sequence variation within *g463* can be due to diversification, which would imply that the target of g463 in onion is also changing and under selection pressure (Allen *et al*., 2004). The screening of *g463* needs to be tested on a broader range of isolates to prove this theory.

In summary, the presence / absence of ten candidate effector genes of Fus2 suggests that the more of these genes are present in a *F. oxysporum* strain it is the more likely to be aggressive in onion seedlings. Collectively these genes may represent the conserved and unique protein-protein and protein-substrate interactions

that assist *F. oxysporum* pathogenicity. PCR-based screening of ten candidate effector genes of Fus2 revealed that *F. oxysporum* ff. spp. *medicaginis* and *asparagi* may have similar lineage specific regions than formae speciales *lycopersici, gladioli* and *dianthi*. Screening the genomes of eleven *F. oxysporum* representing several formae speciales revealed that homologues of ten candidate effector genes of Fus2 are more frequently present in formae speciales *pisi*, *radicis-lycopersici* and *conglutinans* than eight other formae speciales. Formae speciales containing similar sets of candidate effectors are probably using similar strategies to modify host cell structure and function for their advantage. To clarify this, it would be useful to test host specificity of Fus2 strain on a variety of plants, such as *Arabidopsis*, pea, tomato, alfalfa and asparagus. Also, the analysis of these genes *in planta*, so as the disruption and overexpression mutants, is necessary to clarify if they are involved in pathogenicity. This study contains only preliminary results as only ten out of 41 genes were further investigated. Among the remaining 31 genes there are promising candidates that could be useful for further investigations. Additionally, genome sequencing of additional FOC isolates, preferably some of those tested negative for the presence of *SIX* genes, would be highly valuable to identify effectors specifically present in FOC.

4.5.4 Annotation of genes of Fus2 without N-terminal secretion signal peptide

Annotation of 509 genes not present in *F. oxysporum* f. sp. *lycopersici* reveal those are involved in the degradation of plant cell wall, translocation of metabolites, synthesis of enzymes, polyketides, small signalling proteins, transcription factors, proteins involved in fungal growth and pathogenicity. The presence of two non-

secreted proteins predicted among these is particularly interesting. One of them is pisatin demethylase (found in two copies in the genome of Fus2), a protein involved in the detoxification of antifungal pisatin toxin produced by pea plants. This gene was found in *F. solani* and *F. oxysporum* f. sp. *pisi* encoded on small chromosomes and the transfer of this gene into *F. oxysporum* f. sp. *lini* promoted pathogenicity on pea (Coleman *et al*., 2011ab). Further homologues of pisatin-demethylase were found in *F. oxysporum* ff. spp. *dianthi, lini, glycines* and *phaseoli*, but only the one from the bean-pathogen was functioning (Coleman *et al*., 2011ab). These results suggest that this gene was gained through horizontal gene transfer by FOC. It has to be investigated if pisatin demethylase of Fus2 is functioning.

Another gene is gibberellin-β-hydroxylase, which is involved in the synthesis of plant hormone gibberellin. *F. sacchari, F. konzum* and *F. subglutinans* were reported to synthesize gibberellins (Troncoso *et al*., 2010). Gibberellin is a plant hormone involved in the utilisation of starch in germinating seeds and in regulation of sugar signalling pathway (Cadman *et al*., 2006). It was proposed that phytopathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* used effectors to upregulate the transcription of *SWEET* (sucrose effluxer) genes of rice to increase sugar efflux into the apoplast (Chen *et al*., 2012). This could suggest that FOC might be capable of manipulating host-cell sugar metabolism for its advantage.

Several genes involved in toxin production were found, however most of these genes are part of a gene cluster (Zhou and Linz, 1999), therefore individual appearance does not indicate that these toxins (aflatoxins and trichothecenes) would be produced by Fus2. Moreover, *Tri15* is *a* negative regulator of at least some of the trichothecene biosynthetic genes (Alexander *et al*., 2004) and trichothecene 3-o-acetyltransferase is able to modify trichothecenes and reduce their toxicity (Khatibi

et al., 2011). This suggests that Fus2 is capable of competing against trichotechene producing fungi. Some entomopathogenic fungi, such as *Metarhizium anisopliae* and *M. acridum* are capable of producing mosquitocidal toxins (Gao *et al*., 2011). Culture filtrates of *F. oxysporum* and spore treatment by *F. pallidoroseum* were able to control tropical disease vector mosquitos (Singh and Prakash, 2011; Mohanty *et al*., 2008).

A reverse transcriptase was also predicted from the genome of Fus2. It is very likely that this reverse transcriptase is the one found on mitochondrial chromosomes of *F. oxysporum* previously (Kistler *et al*., 1997). The latest research suggests that this reverse transcriptase is capable of utilizing RNA, DNA and protein primers and was suggested to be an evolutionary precursor of a broad range of retroelements, including hepadnaviruses, non-long terminal repeat (non-LTR) retrotransposons and telomerase (Galligan *et al*., 2011).

Two heterokaryon incompatibility proteins were predicted, which is not too surprising as several VCGs of FOC have been found (Southwood *et al*., 2012). The surprising finding is that one of them is actually encoded closely to one of the pisatin demethylase genes. This could suggest that VCGs are useful to distinguish host specific forms of *F. oxysporum*.

These preliminary results suggest that there is key information in terms of host specificity hidden under the avalanche of genome sequence data. For example the presence of pisatin demethylase in the genome of FOC is a sign that FOC could potentially cause diseases on peas or maybe even other legumes. The annotation of the whole genome would provide a better understanding of host-pathogen interaction within *F. oxysporum*.

4.5.5 Evaluation and test bioinformatics tools and markers for the identification of further effector genes

Five pathogenicity genes, *ARG1, FMK1, FOW1*, and *FOXG_00016* of *F. oxysporum* ff. spp. *lycopersici* and *phaseoli,* listed in PHI-BASE database (Winnenburg *et al*., 2008) showed low amino acid similarities to FOC, therefore they could be good candidates for its specific identification. The PHI-BASE have not been updated since 2006, therefore the screening of pathogenicity genes published more recently is essential.

Also, the presence of mimp elements in the promoter region of candidate genes could be a useful marker for the identification of some *SIX* genes in the genome of Fus2.

Comparison of *SIX5* and *SIX3* encoding contigs led to the identification of a 19 bp identical region. It would be useful to investigate whether this short sequence present upstream from other candidate effectors.

A novel motif, L[HF][HS][HS]A, was found among four secreted proteins which could be a useful marker for the prediction of further effector genes one all genes predicted from the genome of Fus2.

Prediction of leucine-rich nuclear export signals (NES) could be extremely valuable as it could predict genes interacting with host nucleus. GFP-labelling of these proteins could clarify whether they target host nuclei.

The presence of motifs does not mean that they are functional; first their location has to be confirmed (N-terminal or C-terminal). For instant, the RXLR-like motifs, [R/K/H]x[L/M/V/F/Y/W]x, is possibly the most important published motif among all screened motifs in oomycetes. It was also demonstrated that RxLR-like motif exist in many fungal effectors including Avr2 (=Six3) of FOL and the motif is

proposed to play an important role in translocation of effectors into the host cell, through binding the phospholipid, phosphatidylinositol-3-phos-phate (PI3P) (Kale *et al*., 2010). However, recent work by Yaeno *et al*. (2011) suggests that PI3P binding of AVR3a of *P. infestans* is mediated by a C-terminal fragment lacking the RXLR domain.

This section showed that there are markers such as motifs and bioinformatics tools available to further characterize the proteome and secretome of FOC and also there are ways to predict ways of transport of effectors into host cell and also the location of the target molecules within host cell.

CHAPTER 5

INTERNAL FRUIT ROT OF SWEET PEPPER IN THE UK

5.1 INTRODUCTION

British sweet pepper (*Capsicum annuum* L.) production has doubled over the last ten years from about 10,000 to 20,000 tonnes with a cash value of £17M annually (Defra, 2011). In the UK, yellow and red varieties are grown mainly in greenhouses and immature green fruits are also sold (Tim O'Neill, ADAS, pers. comm.).

There are several fungal diseases causing economic losses for British sweet pepper growers such as *Phytophthora spp., Alternaria spp. Cercospora capsici, Leveillula taurica, Sclerotium rolfsii* and *Fusarium* species. Two different diseases are caused by *Fusarium* species on pepper fruits: stem and fruit rot and internal fruit rot (Figure 32).

Stem and fruit rot is caused by *Fusarium solani* (teleomorph *Nectria haematococca)* which gives black water-soaked lesions on the stem around the nodes and on the sides of the fruit (Jarvis *et al*., 1994, Fletcher, 1994, Lamb *et al*., 2000, Ramdial & Rampersad, 2010). This disease has been reported in Canada, United Kingdom, Trinidad, Hungary, Venezuela and New Zealand (Jarvis *et al*., 1994, Fletcher, 1994, Ramdial & Rampersad, 2010, Lukacs & Szarka, 1988, Cedeño *et al*., 2003). Contaminated rockwool cubes in which seedlings are grown could be a means of spread of stem and fruit rot of sweet pepper (Smudja, 1992). An increasing problem is internal fruit rot which was first reported in the early 2000s in Canada, Belgium, the Netherlands, the UK and Korea (Yang *et al*., 2009, Van Poucke *et al*., 2012, Choi *et al*., 2011).

Figure 32. Sweet pepper disease symptoms caused by *Fusarium* species. **A-B**. Symptoms of pepper stem and fruit rot (Photo credit: Cerkauskas, R., Agriculture and Agri-Food Canada). **A**. Black lesion around calyx. **B**. External and internal discoloration of stem. **C-D**: Symptoms of internal fruit rot of pepper (Photo credit: Yang, Y., University of Alberta). **C**. Pepper seeds are covered by mycelium. **D**. Brown discoloration of pepper fruit.

Unlike the stem and fruit rots, the symptoms are not as obvious as the rot starts from the inside of the pepper fruit with white mycelium covering the seeds and internal surface of the fruit. In a later stage, brown lesions can be seen on the outer surface of the fruit, which helps to remove the diseased fruits before marketing. Utkhede & Matur (2003) identified a *Fusarium subglutinans*-like fungus as the causal agent of internal fruit rot in 2003. Later, two groups reclassified the causal agent as *Fusarium lactis*, although *Fusarium proliferatum*, *Fusarium oxysporum* and *Fusarium solani* were also isolated from the same diseased fruits (Yang *et al*., 2009, Van Poucke *et al*., 2012). Van Poucke *et al*. (2012) showed that *F. lactis* isolates form a monophyletic clade, *F. lactis* species complex (FLASC), based on the translation elongation factor 1α (EF), β-tubulin (TUB), calmodulin (CAM) and the

second largest subunit of RNA polymerase II (RPB2) gene sequences. Twelve multi locus sequence types (ST1 to ST12) were defined within the *Fusarium lactis* species complex (Table 31). They also proposed that some of these isolates form a new, monophyletic species FLASC-1. *Fusarium lactis* causes fruit rot by infecting the flower through the transmitting tissues of the style and grows on the surface of the seeds (Yang *et al*., 2010).

Table 31. Genetic description of *Fusarium lactis* species complex multilocus sequence types (ST1 to ST12) from sweet pepper published by van Poucke *et al*., 2012.

ST #	No. of isolates	Representative isolates[a]	EF[b]		CAM[c]		*RPB2*[d]	
			group	GenBank accession #	group	GenBank accession #	group	GenBank accession #
ST1	36	MUCL 51511	1	FR870279	1	FR870291	A	FR870303
ST2	13	MUCL 51516	1	FR870280	2	FR870292	B	FR870304
ST3	8	MUCL 51515	1	FR870281	2	FR870293	C	FR870305
ST4	2	MUCL 51523	2	FR870282	3	FR870294	D	FR870306
ST5	3	MUCL 52692	3	FR870283	4	FR870295	E	FR870307
ST6	6	MUCL 52697	3	FR870284	4	FR870296	E	FR870308
ST7	4	MUCL 52695	3	FR870285	4	FR870297	F	FR870309
ST8	1	MUCL 52696	4	FR870286	5	FR870298	B	FR870310
ST9	1	MUCL 52803	5	FR870287	4	FR870299	G	FR870311
ST10	1	NRRL 31629	7	FR870288	6	FR870300	A	FR870312
ST11	1	NRRL 31630	1	FR870289	7	FR870301	A	FR870313
ST12	2	[T] MUCL 51854	6	FR870290	4	FR870302	G	FR870314

[a] MUCL, Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium; [T], neotype
[b] EF, translation elongation factor 1α gene.
[c] CAM, calmodulin gene.
[d] *RPB2*, second largest subunit of RNA polymerase II gene.
[e] TUB, β-tubulin gene.
[f] MAT, mating type *MAT1-1* or *MAT1-2*.
[g] ND: not determined.

It has been shown that some *F. proliferatum* and *F. lactis* isolates were able to produce limited amounts of the mycotoxin beauvericin and traces of fumonisin in pepper (Van Poucke *et al.*, 2012). Beauvericin was detected only in the lesions, while fumonisins were able to migrate to the surrounding, healthy tissue of the sweet pepper. There is a risk that diseased pepper fruits can be consumed as the symptoms are not always can be seen externally and therefore it is difficult to cull unhealthy fruits before delivery to market (Yang *et al*., 2011).

It is not clear how the disease spreads and measures recommended to reduce occurrence of Fusarium internal fruit rot include good sanitation practices, careful disposal of infected fruits and avoiding wounding of the fruit (O'Neill and Barbara, 2012). It has been shown that fusaria spore concentrations in the greenhouse air increased with higher temperature, humidity and radiation, therefore maintaining good air circulation and keeping the relative humidity below 85% helps to manage the disease. In Canada, bumblebees (*Bombus spp*.) are used as pollinators and these can also carry *Fusarium* spores (Yang *et al*., 2009). In the UK, pollinators are rarely used but fruits damaged by the tortrix moth caterpillar showed a high incidence of Fusarium rot (O'Neill and Barbara, 2012). Several chemical and biological treatments have been suggested to control internal fruit rot (Utkhede and Mathur, 2006). Flowers treated with iprodione, *Gliocladium catenulatum* or *Bacillus subtilis* had significantly higher fruit weight compared with controls. Resistant pepper varieties are not available, but varieties that produce small flowers appear more tolerant.

5.2 AIMS AND OBJECTIVES

Detailed aims and objectives of this chapter were:

- To obtain *Fusarium* isolates from sweet pepper
- To characterize isolates from sweet pepper fruit showing internal fruit rot symptoms at species level by using sequences of three molecular markers (*TEF*, *RPB2* and *CAL*)
- Compare sequence data of *Fusarium* isolates associated with internal fruit rot of sweet pepper obtained during this project with data previously published on this disease

5.3 MATERIALS AND METHODS

5.3.1 List of *Fusarium* isolates associated to fruit rot of pepper in the UK

Fusarium isolates (18) obtained from pepper fruit showing internal rot symptoms were characterised based on morphology on PDA by Tim O'Neill (ADAS) (Table 32, O'Neill and Barbara, 2012).

Table 32. Origin and morphology on PDA of 18 *Fusarium* isolates obtained from sweet pepper from three UK nurseries.

Isolate	Source of sample			Colour in culture on PDA
	Nursery (code)	Variety	Tissue	
1	C	Special	Fruit	Peach
2	C	Special	Fruit	Peach
3	C	Special	Fruit	Peach
4	C	Special	Fruit	Peach
5	C	Fiesta	Fruit	Peach
6	C	Special	Stem	Peach
7	A	Ferrari	Fruit	Peach
8	A	Spider	Fruit	White
9	B	Ferrari	Fruit	Purple
10	B	Ferrari	Fruit	White/pink
11	B	Ferrari	Fruit	White
12	B	Ferrari	Fruit	White
13	B	Ferrari	Fruit	Purple
14	B	Kelly	Fruit	White/pinky red
15	B	Kelly	Fruit	White
16	B	Kelly	Fruit	White/pinky red
17	B	Kelly	Fruit	White/pinky red
18	B	Kelly	Fruit	White/pinky red

A,B and C are referring to three different pepper nurseries in the UK

5.3.2 Molecular identification of *Fusarium* isolates obtained from sweet pepper showing internal fruit rot symptoms

DNA was extracted using a Qiagen DNeasy Plant Mini-kit with minor modifications as described in Section 4 (carried out by C. Grant, Warwick University). Three genes were used for the identification of *Fusarium* isolates: *TEF*,

calmodulin and RNA polymerase II subunit (*RPB2*). *TEF* targeting primers used were designed in-house (Table 7), while primer pairs amplifying *RPB2* and calmodulin were identical to those used in a publication (O'Donnell *et al*. 1998a, 2000, 2007). PCR reactions were as in Section 3.3.1.3 and sequencing of clean products was done as in Section 3.3.1.3.

5.3.2 Cladistic analysis of *Fusarium* isolates obtained from sweet pepper showing internal fruit rot symptoms

Sequence alignment and cladistics analysis was done as in Section 3.3.3.3. Additionally, for taxonomic reference *TEF* (26 sequences), calmodulin (19 sequences) and *RPB2* (17 sequences) sequences downloaded from NCBI were included in the cladistics analysis. Individual cladograms were produced based on *TEF, RPB2* and calmodulin sequences. Each cladograms contained sequences corresponding to a *F. lactis* type isolate recovered from fig fruit (Nirenberg and O'Donnell, 1998).

5.4 RESULTS

Fusarium species were not clearly distinguishable based on their morphology on PDA. Peach colonies were identified as *F. proliferatum* and *F. lactis*-like species, white colonies as *F. proliferatum F. solani* and *F. oxysporum*, purple colonies as *F. solani* and *F. lactis*-like species. Only the white/pinky-red colonies were identified uniformly as representatives of *F. oxysporum*.

Analysis of partial *TEF*, calmodulin and *RPB2* gene sequences led to the identification of *F. oxysporum* (6 isolates), *F. proliferatum* (3 isolates), *F. solani* (2 isolates) and *F. lactis* (7 isolates) species (Table 33). Analysis of partial *TEF*

sequences showed that *F. oxysporum*, *F. proliferatum* and *F. solani* isolates formed distinct clades which corresponded to the different *Fusarium* species used as taxonomic references. The topology of the *TEF* cladogram was confirmed by *RPB2* and calmodulin sequences (Figures 33 - 35).

British *F. lactis* isolates formed two clades, based on data from all three loci and showed high sequence similarity (>98%) to a type isolate recovered from fig fruit (Table 33). Six isolates (isolates 1-6) formed a distinct clade with some Belgian and Korean *F. lactis* isolates collected from sweet pepper showing internal fruit rot. Isolate 13 and a few Belgian isolates also formed a distinct clade. The presence of these two clades was confirmed by *RPB2* and calmodulin sequences.

All three isolates from pepper belonging to *F. proliferatum* were identical based on the sequence of all three molecular markers (Table 33). Similarly, there was no variation in sequence between British *F. lactis* isolates in clade 1 (isolates 1 - 6). Sequence comparison of our isolates to the isolates' used in a Belgian study (van Poucke *et al*., 2012) revealed that clade 1 corresponds to sequence type 1 (ST1) and clade 2 to ST7 defined by this group (Table 33). Several different sequence types were represented within clades corresponding to *F. oxysporum* and *F. solani*.

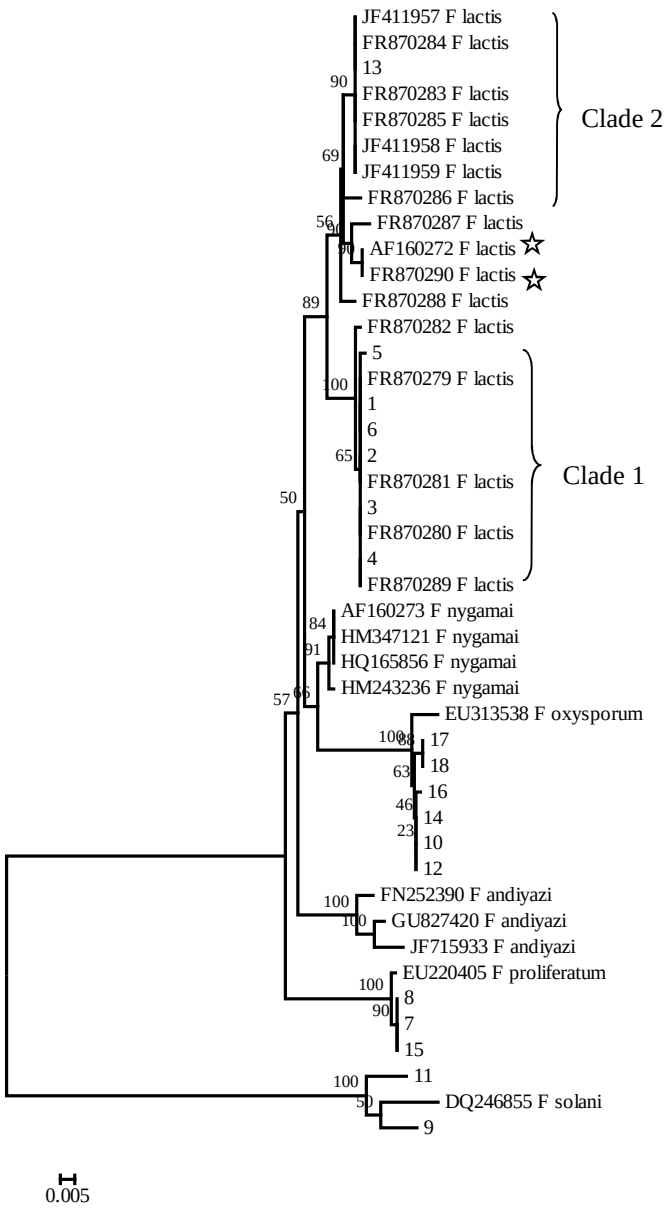


Figure 33. Neighbour-joining cladogram of representatives of *Fusarium* species associated with sweet pepper based on partial translational elongation factor 1α (*TEF*) dataset. Consensus tree is shown with bootstrap values from 1000 replicates. GenBank accession number is indicated for each reference sequence. ☆: *Fusarium lactis* type isolates from *Ficus* sp. Accession numbers starting with FR and JF refer to *Fusarium* isolates recently recovered from sweet pepper grown in Belgium and Korea, respectively.

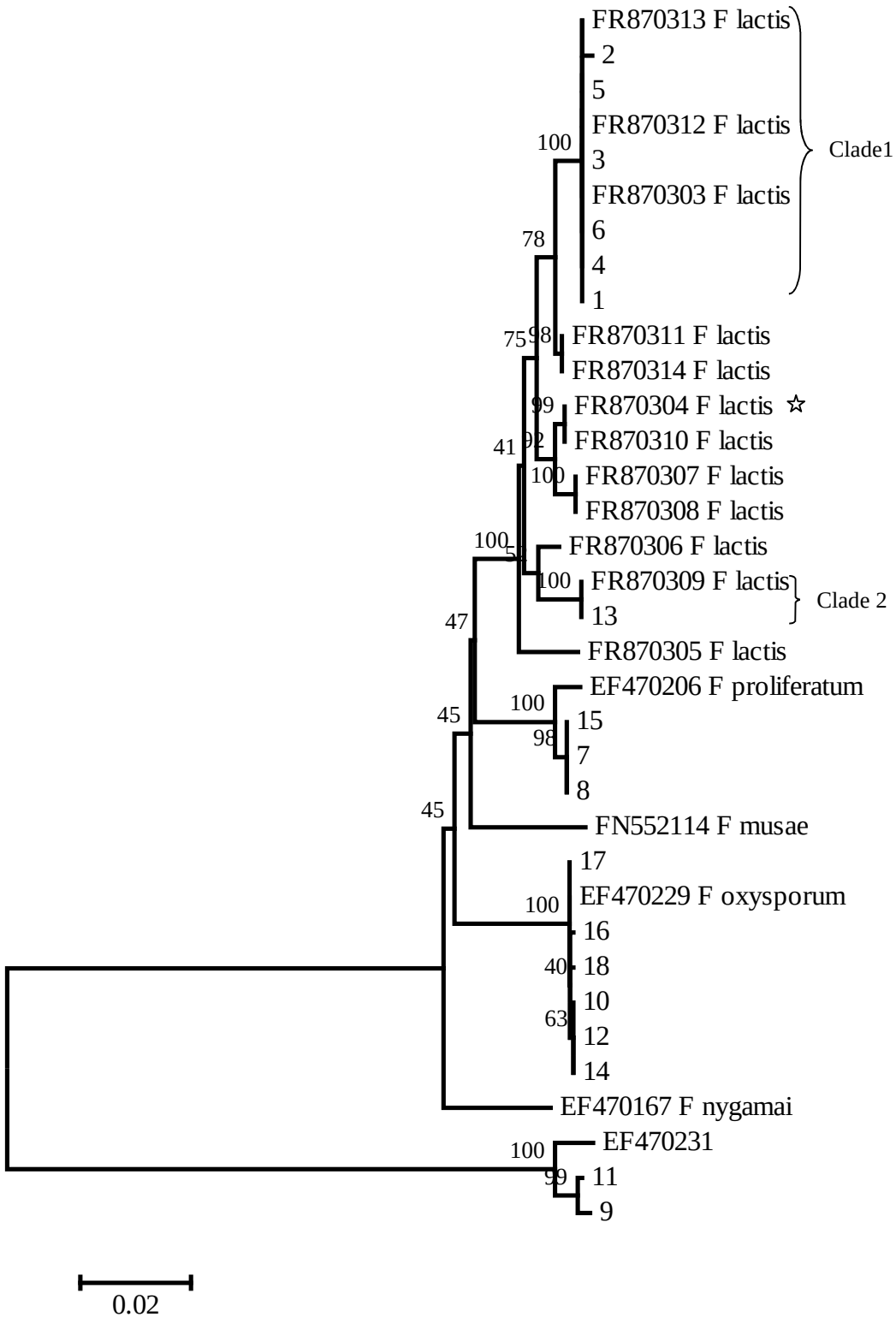


Figure 34. Neighbour-joining cladogram of representatives of *Fusarium* species associated with sweet pepper based on partial RPB2 dataset. Consensus tree is shown with bootstrap values from 1000 replicates. GenBank accession number is indicated for each reference sequence. Accession numbers start with FR refer to *Fusarium* isolates recently recovered from sweet pepper from Belgium. ☆ : *F. lactis* type isolate from *Ficus* sp.

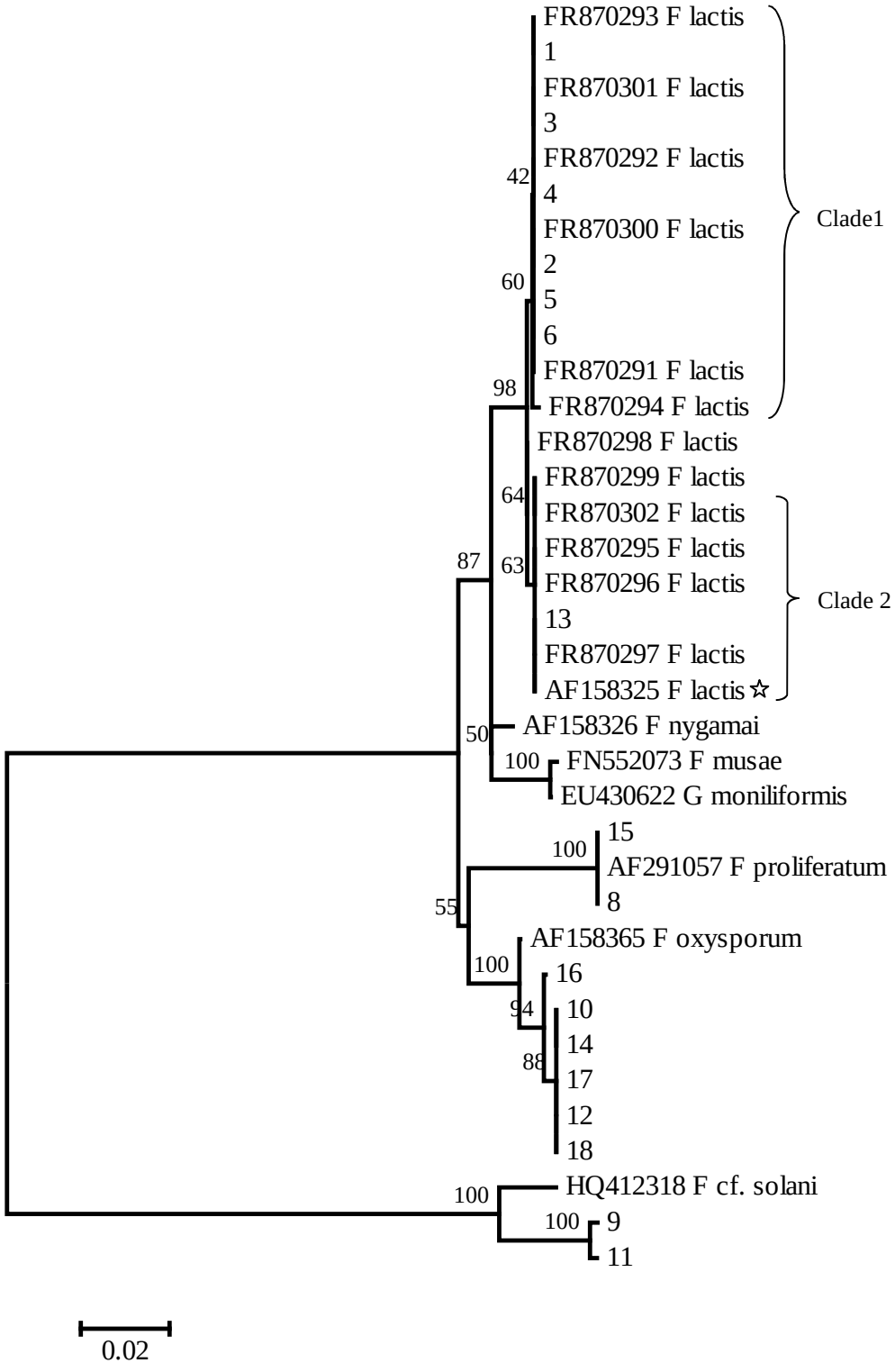


Figure 35. Neighbour-joining cladogram of representatives of *Fusarium* species associated with sweet pepper based on partial calmodulin dataset. Consensus tree is shown with bootstrap values from 1000 replicates. Phylogenetic analysis was conducted in MEGA 4. GenBank accession number is indicated for each reference sequence. ☆ *Fusarium lactis* type isolate from *Ficus* sp. Accession no start with FR refer to *Fusarium* isolates recovered from pepper.

Table 33. Description of *Fusarium* isolates obtained from British greenhouse pepper based on morphology and multi locus sequence similarity.

WU ref	Nursery	Colony colour on PDA	TEF	RPB2	CAL	Identification
1.	C	Peach	1	A	1	*F. lactis*
2.	C	Peach	1	A	1	*F. lactis*
3.	C	Peach	1	A	1	*F. lactis*
4.	C	Peach	1	A	1	*F. lactis*
5.	C	Peach	1	A	1	*F. lactis*
6.	C	Peach	1	A	1	*F. lactis*
7.	A	Peach	FP	FP	FP	*F. proliferatum*
8.	A	White	FP	FP	FP	*F. proliferatum*
9.	B	Purple	FS1	FS1	FS1	*F. solani*
10.	B	White/pink	FO1	FO1	FO1	*F. oxysporum*
11.	B	White	FS2	FS2	FS2	*F. solani*
12.	B	White	FO1	FO1	FO1	*F. oxysporum*
13.	B	Purple	3	F	4	*F. lactis*
14.	B	White/pinky red	FO1	FO1	FO1	*F. oxysporum*
15.	B	White	FP	FP	FP	*F. proliferatum*
16.	B	White/pinky red	FO2	FO1	FO2	*F. oxysporum*
17.	B	White/pinky red	FO3	FO1	FO1	*F. oxysporum*
18.	B	White/pinky red	FO3	FO1	FO1	*F. oxysporum*

All isolates from fruit except isolate 9 (from a stem lesion).
TEF: TEF sequence group, RPB2: RPB2 sequence group, CAL: calmodulin sequence group
Sequence groups definition was adapted for *F. lactis* isolates from van Poucke *et al*. (2012).

Two isolates recovered from peppers grown in nursery A belonged to *F. proliferatum*, while all six isolates recovered from nursery C were identified as representatives of clade 1 within *F. lactis*. All four *Fusarium* species (including *F. solani* and *F. oxysporum*) were recovered from nursery B.

SUMMARY

Fusarium isolates (18) recovered from British sweet peppers showing internal fruit rot symptoms from three nurseries were identified based on partial *TEF*, calmodulin and *RPB2* gene sequences. This led to the identification of *F. oxysporum*

(6 isolates), *F. proliferatum* (3 isolates), *F. solani* (2 isolates) and *F. lactis* (7 isolates) species. *F. lactis* isolates formed two clades, based on data from all three loci and showed high sequence similarity (>98%) to a type isolate recovered from fig fruit. Sequence comparison of our isolates to the isolates' used in a Belgian study (van Poucke *et al*., 2012) revealed that clade 1 corresponds to sequence type 1 (ST1) and clade 2 to ST7 defined by this group. All three isolates from pepper belonging to *F. proliferatum* were identical based on the sequence of all three molecular markers, while several different sequence types were represented among *F. oxysporum* and *F. solani* isolates.

5.5 DISCUSSION

Internal fruit rot of sweet pepper appeared about ten years ago in the UK and has been an increasing problem since then (Tim O'Neill, ADAS, pers. comm.). As the symptoms are not always visible on the surface of the pepper fruit, it is difficult to cull diseased plants before they are marketed. A study in Canada revealed that the infection is caused by *F. lactis* which infects the flower following pollination (Yang *et al*., 2010).

The majority of the *Fusarium* isolates (seven out of 18 isolates) collected from pepper showing internal fruit rot symptoms were identified as *F. lactis*, which was reported as the principal cause of internal fruit rot in Canada and Korea (Yang *et al*. 2009, Choi *et al*., 2011). In an extensive study in Belgium, most of the isolates were identified as "*F. lactis*-like" species (van Poucke *et al*., 2012). The same study also included British, Belgian, Dutch and Canadian isolates from pepper and demonstrated significant genetic variation among the *F. lactis*-like isolates. Twelve sequence types (STs) were defined among *F. lactis*-like isolates and all three British isolates included in this study fell into ST1, the most common type. The majority of recently obtained isolates (isolates 1 - 6) also belonged to ST1, with one exception. A representative of ST7 (isolate 13) was recovered from nursery B. Presence of representatives of ST7 had not been reported from the UK previously. *F. lactis* was described as causal agent of endosepsis of fig in California (Nirenberg and O'Donnell, 1998) and since then these isolates are used as taxonomic references. Based on three loci (*TEF, RPB2* and calmodulin) our *F. lactis*-like isolates show at least 98% similarity to the type isolates from fig which shows high intraspecific variation, *i. e*. twelve multiple sequence types.

F. oxysporum was the second most common species isolated from British pepper showing internal fruit rot symptoms. Previous studies also detected *F. oxysporum*, but at a lower frequency compared to *F. lactis* (Yang *et al*., 2009, van Poucke *et al*., 2012). *F. oxysporum* has been reported in association with internal fruit rot and also with stem and fruit rot of British greenhouse pepper (O'Neill, 2008). The first symptom of wilting is usually yellowing of the leaves, which was not observed on plants with internal fruit rot. It is more likely that *F. oxysporum* is a secondary invader of the diseased fruit. The other possibility is that *F. oxysporum* also capable of infecting the plant through the flower, but this has not been tested.

A few *F. proliferatum* isolates were also present in British pepper showing internal fruit rot symptoms, which in in agreement with previous publications from Canada and Belgium (Yang *et al*., 2009, van Poucke *et al*., 2012). *F. proliferatum* is capable of causing "internal infection" on pepper fruits (Yang *et al*., 2009), but has not been reported in association with internal rot of sweet pepper from the UK previously.

F. solani was the least common species found on British sweet pepper showing internal fruit rot symptoms, which is in agreement with previous findings (van Poucke *et al*., 2012). *F. solani* was previously has been reported as the causal agent of stem and fruit rot of pepper in the UK (Fletcher, 1994). 18 years ago this disease affected less than 1 % of the crop in the UK, although, *F. solani* caused a more rapid external rot on pepper fruits (Yang *et al*, 2009).

Our isolates were collected from three nurseries (A, B and C) in the UK (same grower), but *F. lactis* was found to be dominant in only one of them (nursery C) and was not present at all in the sample from another nursery (A). The third nursery (B) had a very diverse community of *Fusarium* species. The present study is

based on 18 isolates, and hence only preliminary conclusions are drawn. More recent screening of aborted sweet pepper fruits suggested that *F. lactis* is the major cause of internal fruit rot in the UK (C. Grant, University of Warwick, pers. comm.).

Analysis based on colony morphology of isolates on PDA was unable to clearly distinguish different *Fusarium* species. Sequence analysis of housekeeping genes (*TEF, RPB2* and calmodulin) was necessary for the identification of *F. lactis* isolates as previous studies showed that *F. verticillioides*- and *F. subglutinans*-specific primers were not able to resolve the species level identification of *F. lactis* isolates.

This small study shows that methods developed for the identification of *Fusarium* species associated with *Allium* species could be employed to study other *Fusarium*-plant pathosystems such as internal fruit rot of sweet pepper. More importantly, the presence of *F. lactis and F. oxysporum* from sweet pepper showing internal fruit rot symptoms in the UK has been confirmed. Also, this is the first report of *F. proliferatum* and a new sequence type (ST7) of *F. lactis* in association with this disease in the UK. Sequence analysis of housekeeping genes proved to be a rapid and reliable method for identifying *Fusarium* species associated with internal fruit rot of sweet pepper.

CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTIVE

6.1 Conclusions

The *Fusarium* genus is taxonomically complex, and comprises a wide variety of filamentous fungi recognised as plant, animal and human pathogens and soil saprophytes. One of the most important member of this genus is *Fusarium oxysporum* Schlecht., includes plant pathogenic (formae speciales) and morphologically indistinguishable non-pathogenic forms that could act as disease suppressors, and it is not uncommon for both to co-exist in cropping-systems.

Allium and sweet pepper crops have been suffering more and more in recent years from diseases caused by *Fusarium* species in the UK. *F. oxysporum* f. sp. *cepae* was identified as causal agent of onion basal rot in the UK twenty years ago and causing increasing problems ever since then, while *F. lactis* was isolated from sweet peppers showing internal fruit rot symptoms only last year. Recent publications reported the co-existence of several *Fusarium* species in both pathosystems in several other countries (Galvan *et al.*, 2008; Bayraktar *et al.*, 2010; Yang *et al.*, 2009; van Poucke *et al.*, 2012). In this thesis, the diversity and the species and sub-species level identity of *Fusarium* species populations associated with diseased *Allium* and sweet pepper crops in the UK was characterised by biotypic and genotypic analysis. An attempt was made to identify molecular markers suitable for the development of a sensitive PCR diagnostic assay for detecting pathogenic *F. oxysporum* f. sp. *cepae* and distinguish these from non-pathogenic isolates and to understand pathogenicity in this fungus.

An important finding of this study was that *F. proliferatum* was found for the first time in the UK in onions with basal rot symptoms, and *F. oxysporum* was the most commonly (80%) isolated species from onion bulbs collected between the 2008

and 2010 in the UK. It was also confirmed that *F. oxysporum* and *F. proliferatum* was pathogenic on onion, although there was variation in aggressiveness. *F. solani* and *F. redolens* were found on onions grown in the UK as frequently as *F. proliferatum* (~5%), but the results of the onion seedling assay suggested that they are not causal agents of onion basal rot in the UK. Although, this needs to be confirmed by using a set of isolates more representative for these species.

More importantly, this is the first evidence that pathogenic isolates of *Fusarium* species on imported onion sets and dry onion bulbs shows can be introduced to the UK. These results suggest that new outbreaks of *Fusarium* might be caused by the import of diseased onion sets, and hence suggest that closer inspection of such planting material is required.

Moreover, this thesis contains the first report of *F. culmorum* and *F. avenaceum* from greenhouse-grown leek showing crown and basal rot symptoms and also the first evidence of *F. proliferatum* and a new sequence type (ST7) of *F. lactis* in association with internal fruit rot of sweet pepper in the UK. Although, the role of these species in disease development is yet to be confirmed.

Previous studies gave a good indication of the diversity of the worldwide population of *F. oxysporum* f. sp. *cepae* (FOC), but none of these included UK isolates (Galvan *et al*., 2008; Swift *et al*., 2002; Bayraktar *et al*., 2011; Southwood *et al*., 2012). Cladistic analysis based on translational elongation factor (*TEF*) sequences of *F. oxysporum* f. sp. *cepae* isolates from various locations with reference to other formae speciales confirmed that British isolates formed two main clades, Clade 2 and Clade 3 having been determined by O'Donnell *et al*. (1998a). Results of the sequence analysis of *TEF* suggest that *F. oxysporum* isolates associated with onion basal rot in the UK are composed of at least nine different sequence types

(STs), but only three of them (FoA, FoB and FoD) were found aggressive in onion seedlings. Isolates classified as belonging to sequence types (STs) FoA, FoB and FoD cluster closely, suggesting a clonal origin of these groups. The diversity of isolates from *Allium* species indicates the polyphyletic origin of FOC, suggesting that pathogenic ability may have evolved convergently.

A set of *F. oxysporum* isolates representing *F. oxysporum* ff. spp. *cepae, lycopersici, pisi, medicaginis, asparagi, freesia, dianthi, gladioli* along with non-aggressive isolates recovered from onion and biocontrol strain Fo47 were further characterised using four housekeeping genes and six microsatellites. Unfortunately, only some of the sequence types or microsatellite patterns could be linked to aggressiveness in onion seedlings, therefore they were not suitable molecular markers for the development for detecting FOC. The set of isolates used in this study provides an invaluable culture collection applicable for future development for breeding resistant onion varieties and to be used in studies on understanding the host specificity of this fungus.

Previous studies reported the presence of effector genes in three formae speciales, which provided a robust identification method (Lievens *et al*., 2009, Chakrabarti *et al*., 2011; Meldrum *et al*., 2012). This is the first report of the discovery of a homologue of an effector gene, *SIX7*, in FOC. Extensive screening of *F. oxysporum* isolates from *Allium* crops for the presence of *SIX7* homologues led to the discovery of *SIX7*'s limitation to one clonal lineage of FOC, the most commonly occurring clonal lineage FoA (78%). Primers developed based on polymorphism in the *SIX7* homologue of FOC (*Foc-SIX7*) offer an opportunity for the specific detection of this group, although *Foc-SIX7* was present in most isolates belonging to

FoA, but not all. This suggests that *Foc-SIX7* is not necessary for pathogenicity of FOC.

Latest publications often use whole genome sequencing to discover homologues of published effector genes and or candidate effector genes (Haas *et al*., 2009; Baxter *et al*., 2010). To date twelve genome sequences of *F. oxysporum* have been published (Broad Institute, Thatcher *et al.*, 2012). This is the first genome sequence of a FOC isolate (Fus2), which provides a novel resource to enhance our understanding of the *F. oxysporum* – non-model plant interaction. FOC genome led to the discovery of the homologues of *SIX3*, *SIX5, SIX7, SIX9* (effector genes) and pisatin-demethylase (virulence gene). This is the first report of the discovery of a homologue of *Fol-AVR2* (*=Fol-SIX3*) and *Fol-SIX5* in FOC Fus2, another f. sp. than *F. oxysporum* f. sp. *lycopersici* (FOL). Using bioinformatics tools a set of genes coding secreted proteins were identified in the genome of FOC Fus2 which are absent in the genome of tomato pathogen FOL 4287. These genes are predicted to be involved in degradation of plant material, chitin synthesis or have unknown function. Screening of ten of these candidate effector genes among a set of isolates representing various formae speciales identified suggests that the more of these genes are present in a *F. oxysporum* isolate it is the more likely to be aggressive in onion seedlings, which may imply that FOC lineages may also have different pathogenic properties. One candidate effector gene (g463) of FOC Fus2 with unknown function was found promising for the specific identification of FOC isolates based on this preliminary data.

6.2 Future prospective

A promising molecular marker, *g463*, has been identified based on the analysis of genome sequence of FOC Fus2 and initial screening of 21 *F. oxysporum* isolates from diseased *Allium* species and different formae speciales. These results have to be confirmed by testing a wider range of *F. oxysporum* isolates. In case this marker found a robust way for identification of FOC, specific markers could be designed to be tested. Primers could be used for expression analysis of this gene during infection of onion, and also from infested soil, dormant onion bulb, seedlings and during grown *in vitro*. For example, TaqMan based method for the detection of FOC in soil and planting material could greatly benefit onion growers in addition to plant inspection authorities. The role of *g463* could be further investigated by deletion and over expression of this gene. Similarly, the role of homologous *SIX7*, *SIX3* and *SIX5* of FOC needs to be clarified. It would be interesting to see in case these genes are functioning in FOC whether they could be exchanged with *SIX* genes of FOL and *vice versa*.

Annotated genome sequencing of further FOC isolates could reveal the presence of further effector genes, especially in those FOC isolates which lack published *SIX* genes. Interestingly, tomato suffers from two diseases caused by two different formae speciales, namely *F. oxysporum* ff. spp. *lycopersici* and *radicis-lycopersici.* The *SIX* genes have only been found from FOL (Broad Institute, Lievens *et al*., 2009a; Menzies *et al.*, 1990). This suggests there is a possibility that there are two formae speciales present on onion. To clarify this, GFP-labelled isolates of each forma specialis could be engineered to investigate how and which organ or tissue of the host are infected and colonised.

Bioassays defining host specificity of FOC isolates could reveal that they possess a threat to and other crops or that FOC could survive and spread by infecting other hosts, such as weed species (Abawi and Lorbeer, 1972). Based on genome sequence data of FOC Fus2, tomato, pea, alfalfa, asparagus and *Arabidopsis* could be good candidates for initial screening. Host specificity of FOC would greatly enhance our understanding on the pathogenicity, epidemiology and maybe even evolution of this fungus or pathogenicity related genes and chromosomes.

Effectors, enzymes and transcription factors important for the pathogenicity of FOC could be found by transcriptomic analysis of total RNA of FOC grown *in vitro* and during infection. This method might help to find resistance genes of onion. Moreover, the comparison of epigenetic differences to differences in pathogenicity could also help us to understand why some isolates having *SIX* genes were not-aggressive in onion seedlings.

Molecular markers applicable for the detection and identification of FOC could help the investigation of the connection of disease severity and amount of FOC propagules or the expression of pathogenicity related genes in soil, onion, crops grown in rotation with onion and weed species.

Molecular markers developed during this work provide a quick and reliable identification of *Fusarium* without any prior knowledge of *Fusarium* taxonomy. For example, *Fusarium* species associated with internal fruit rot of pepper were identified by using a newly developed primer pair targeting *TEF* (exTEFfor/FUexTEFrev). The cost and time of this work could be further reduced by using *Fusarium* species-specific primer pairs. *F. lactis*-specific markers have not been published, but by utilizing the multiple sequence data generated during this work and being generated (by C. Grant, Warwick University) could be useful to

design *F. lactis* specific primers. Additionally, it would be helpful to clarify the pathogenicity of *Fusarium* species associated with internal fruit rot in the UK and compare results of this with multiple sequence data generated. This way the economically devastating *Fusarium* species could be identified from environmental samples, such as planting material, hydroponic system and rock wool.

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APPENDIX I

Table 34. Name, host, origin and source of cultures used for taxonomic reference.

Isolates	Host	Origin	Source
Fusarium oxysporum f. sp. *narcissi*			
B23	*Narcissus* sp.	UK (Lincolnshire)	Carder, J.
N15	*Narcissus* sp.	UK (Lincolnshire)	Carder, J.
Fusarium oxysporum f. sp. *dianthi*			
R207	*Dianthus* sp.	n. i.	Carder, J.
NRRL 26222	*Dianthus* sp.	Netherlands	ARS
Fusarium oxysporum f. sp. *tulipae*			
CBS 242.59	*Tulipa* sp.	Germany	Carder, J.
Ga2	*Tulipa* sp.	n. i.	Bergstrom, G. C.
Fusarium oxysporum f. sp. *lycopersici*			
FOL Race 1	*Solanum lycopersicum*	n. i.	Carder, J.
FOL Race 2	*Solanum lycopersicum*	n. i.	Carder, J.
NRRL 22544	*Solanum lycopersicum*	n. i.	ARS
NRRL 36425	*Solanum lycopersicum*	Netherlands	ARS
Fusarium oxysporum f. sp. *cubense*			
ATCC 96290	*Musa acuminata*	Australia (Queensland)	ATCC
Fusarium oxysporum f. sp. *phaseoli*			
ATCC 90245	*Phaseolus vulgaris*	USA (Colorado)	Carder, J.
Fusarium oxysporum f. sp. *pisi*			
FOP Race 1	*Pisum sativum*	n. i.	Carder, J.
FOP Race 2	*Pisum sativum*	n. i.	Carder, J.
FOP Race 5	*Pisum sativum*	n. i.	Carder, J.
NRRL 36311	*Pisum sativum*	Netherlands	ARS
Fusarium oxysporum f. sp. *cepae*			
Fus2	*Allium cepa*	UK (Lincolnshire)	Noble, R.
NRRL 22538	*Allium cepa*	Germany	ARS
25	*Allium cepa*	USA (Colorado)	Schwartz, H.
22	*Allium fistulosum*	Japan	Ito, S.
Fusarium oxysporum f. sp. *cepae*			
18	*Allium fistulosum*	Japan	Ito, S.
18B	*Allium porrum*	Australia	Hall, B.H.
PR5	*Allium porrum*	Italy	Gilardi, G.
PR7	*Allium porrum*	Italy	Gilardi, G.
Fusarium oxysporum f. sp. *asparagi*			
FOA4	*Asparagus officinalis*	Spain	Molinero-Ruiz, L.
FOA5	*Asparagus officinalis*	Spain	Molinero-Ruiz, L.
Fusarium oxysporum f. sp. *medicaginis*			
Fom004	*Medicago sativa*	USA	Bergstrom, G. C.
Fusarium oxysporum f. sp. *gladioli*			

NRRL 26993	*Gladiolus* sp.	Netherlands	ARS
Fusarium oxysporum f. sp. *freesia*			
NRRL 26990	*Freesia* sp.	Netherlands	ARS
NRRL 26988	*Freesia* sp.	Netherlands	ARS
Fusarium oxysporum			
FOB	*Allium cepa*	Ireland	Grogan, H.
Fus1	*Allium cepa*	UK (Nottinghamshire)	Noble, R.
Fus3	*Allium cepa*	UK (Nottinghamshire)	Noble, R.
151	*Allium cepa*	Netherlands	Koenraadt, H.
180	*Allium cepa*	Netherlands	Koenraadt, H.
181	*Allium cepa*	Netherlands	Koenraadt, H.
PG	*Allium cepa*	UK (Cambridgeshire)	O'Neill, T.
FO47	Soil (biocontrol agent)	France	ARS
Fusarium equiseti			
C-3-1-2	*Brassica napus*	UK (Norfolk)	Atwood, R.
Fusarium proliferatum			
FOA	*Allium cepa*	Ireland	Grogan, H.
IMI 202873	*Cattleya* hybrid	Germany	Carder, J.
31	*Allium cepa*	USA (Colorado)	Schwartz, H.
50	*Allium cepa*	USA (Colorado)	Schwartz, H.
Fusarium avenaceum			
2308-1b	*Solanum tuberosum*	UK (Scotland)	Peters, J.
C1	*Allium cepa*	Ireland	Grogan, H.
Fusarium solani			
R161 AF4	*Pisum sativum*		Carder, J.
Fusarium culmorum			
C-14-2-5	*Brassica napus*	UK (Norfolk)	Atwood, R.
Fusarium verticillioides			
MPVP 294	*Zea mays*	Northern Italy	Magan, N.
Fusarium redolens			
A-18-2-5	*Brassica napus*	UK (Norfolk)	Atwood, R.
Fusarium fujikuroi			
IMI 202879	*Oryza sativa*	Taiwan	CABI
Fusarium succisae			
IMI 202876	*Succisa pratensis*	Germany	CABI
Fusarium coeruleum			
2201-5c	*Solanum tuberosum*	UK (Scotland)	Peters, J.
Fusarium sambucinum			
2504-1c	*Solanum tuberosum*	UK (Scotland)	Peters, J.
Fusarium torulosum			
102	n. i.	n. i.	Lane, C. R.
Fusarium poae			
710	n. i.	n. i.	Lane, C. R.

Fusarium begoniae			
775	n. i.	n. i.	Lane, C. R.
Fusarium tricinctum			
817	n. i.	n. i.	Lane, C. R.
Fusarium cerealis			
831	n. i.	n. i.	Lane, C. R.
Fusarium graminearum sensu lato			
1489	*Cymbopogon* sp.	South Africa	Lane, C. R.
Fusarium foetens			
2076	*Begonia* sp.	Netherlands	Lane, C. R.
Microdochium nivale			
08251	n. i.	n. i.	Noble, R.
Colletotrichum gloeosporioides			
Apple3	*Malus* sp.	UK	Sreenivasaprasad, S.

n. i. : No information available.

Carder, J. (University of Warwick), ARS - Agricultural Research Service, Bergstrom, G. C. (Cornell University), ATCC - American Type Culture Collection, Noble, R. (East Malling Research), Schwartz, H. (Colorado State University), Ito, S. (Yamaguchi University), Hall, B.H. (SARDI), Gilardi, G. (University of Torino), Molinero-Ruiz, L. (University of Cordoba), Grogan, H. (Teagasc), Koenraadt, H. (Naktuinbouw), O'Neill, T. (ADAS), Atwood, R. (University of Warwick). Peters, J. (FERA), Magan, N. (Cranfield University), CABI - Centre for Agricultural Bioscience International , Lane, C. R. (FERA) Sreenivasaprasad, S. (University of Bedfordshire)

APPENDIX II

Table 35. Distribution of *Fusarium oxysporum* isolates associated with *Allium* species according to host, origin, year of collection *TEF* sequence type and presence of *SIX7* gene.

Name	Host	Collected	Origin	Year	TEF	SIX7
18	Welsh o.	n.i.	Japan	2006	F	neg
22	Welsh o.	n.i.	Japan	2006	F	neg
PR5	leek	n.i.	Italy	2005	H	pos
PR7	leek	n.i.	Italy	2005	B	neg
18B	leek	n.i.	Australia	2000	A	neg
Gr2	garlic	stored	Bedfordshire, UK	2010	B	neg
GR4	garlic	stored	Bedfordshire, UK	2010	A	neg
GR4-2	garlic	stored	Bedfordshire, UK	2010	A	neg
Gr5-1	garlic	stored	Bedfordshire, UK	2010	E	neg
GR5-2	garlic	stored	Bedfordshire, UK	2010	E	neg
Gr5-3	garlic	stored	Bedfordshire, UK	2010	E	neg
Sh1-1	shallot	stored	Bedfordshire, UK	2010	D	neg
Sh3	shallot	stored	Bedfordshire, UK	2010	D	neg
Sh4	shallot	stored	Bedfordshire, UK	2010	B	neg
A1	onion	stored	Field 3, Bedfordshire,UK	2009	I	neg
A10	onion	stored	Field 3, Bedfordshire,UK	2009	I	neg
A12	onion	stored	Field 3, Bedfordshire,UK	2009	I	neg
A13	onion	stored	Field 3, Bedfordshire,UK	2009	I	neg
A24	onion	stored	Field 2, Bedfordshire, UK	2009	A	pos
A23	onion	stored	Field 2, Bedfordshire, UK	2009	A	pos
A25	onion	stored	Field 2, Bedfordshire, UK	2009	A	pos
A26	onion	stored	Field 2, Bedfordshire, UK	2009	B	neg
A27	onion	stored	Field 2, Bedfordshire, UK	2009	A	pos
A28	onion	stored	Field 2, Bedfordshire, UK	2009	B	neg
A7	onion	stored	Field 4, Bedfordshire, UK	2009	A	pos
A29	onion	stored	Field 4, Bedfordshire, UK	2009	A	pos
A32	onion	stored	Field 4, Bedfordshire, UK	2009	A	pos
A35	onion	stored	Field 4, Bedfordshire, UK	2009	A	pos
A38	onion	stored	Field 4, Bedfordshire, UK	2009	A	pos
F1	onion	stored	Field 1, Bedfordshire, UK	2010	A	pos
F2	onion	stored	Field 1, Bedfordshire, UK	2010	A	pos
F3	onion	stored	Field 1, Bedfordshire, UK	2010	A	pos
F4	onion	stored	Field 1, Bedfordshire, UK	2010	A	pos
A21	onion	stored	Field 9, Suffolk, UK	2009	A	pos
A3	onion	stored	Field 9, Suffolk, UK	2009	A	pos
A36	onion	stored	Field 9, Suffolk, UK	2009	A	pos
A63	onion	field	Field 9, Suffolk, UK	2009	A	pos
A60	onion	field	Field 9, Suffolk, UK	2009	A	pos
A62	onion	field	Field 9, Suffolk, UK	2009	A	pos
R3	onion	stored	Field 10, Suffolk, UK	2009	G	neg
M1	onion	stored	Field 13, Suffolk, UK	2010	A	pos
M2	onion	stored	Field 13, Suffolk, UK	2010	A	pos
M3	onion	stored	Field 13, Suffolk, UK	2010	A	pos
M6	onion	stored	Field 13, Suffolk, UK	2010	A	pos
M7	onion	stored	Field 13, Suffolk, UK	2010	A	pos

M8	onion	stored	Field 13, Suffolk, UK	2010	J	neg
M9	onion	stored	Field 13, Suffolk, UK	2010	J	neg
G12	onion	field	Field 12, Suffolk, UK	2009	L	neg
K2	onion	stored	Field 11, Suffolk, UK	2009	A	pos
K3b	onion	stored	Field 11, Suffolk, UK	2009	A	pos
P2B-1	onion	stored	Field 5, Essex, UK	2009	A	pos
S1B	onion	stored	Field 5, Essex, UK	2009	A	pos
A14	onion	stored	Field 6, Essex, UK	2009	A	pos
A15	onion	stored	Field 6, Essex, UK	2009	A	pos
A16	onion	stored	Field 6, Essex, UK	2009	A	pos
A17	onion	stored	Field 6, Essex, UK	2009	A	pos
A18	onion	stored	Field 6, Essex, UK	2009	A	pos
A19	onion	stored	Field 6, Essex, UK	2009	A	pos
NL6	onion	field	Field 8, Essex, UK	2010	A	pos
NL8	onion	field	Field 8, Essex, UK	2010	A	pos
NL9	onion	field	Field 8, Essex, UK	2010	A	pos
NL10	onion	field	Field 8, Essex, UK	2010	A	pos
NL11	onion	field	Field 8, Essex, UK	2010	A	pos
NL20	onion	field	Field 8, Essex, UK	2010	A	pos
NL21	onion	field	Field 8, Essex, UK	2010	A	pos
NL22	onion	field	Field 8, Essex, UK	2010	A	pos
NL24	onion	field	Field 8, Essex, UK	2010	A	pos
NL25	onion	field	Field 8, Essex, UK	2010	A	pos
NL26	onion	field	Field 8, Essex, UK	2010	A	pos
NL27	onion	field	Field 8, Essex, UK	2010	A	pos
NL28	onion	field	Field 8, Essex, UK	2010	A	pos
NL32	onion	field	Field 8, Essex, UK	2010	A	n.i.
NL34	onion	field	Field 8, Essex, UK	2010	A	pos
NL36	onion	field	Field 8, Essex, UK	2010	A	n.i.
NL37	onion	field	Field 8, Essex, UK	2010	A	pos
NL42	onion	field	Field 8, Essex, UK	2010	A	pos
NL44	onion	field	Field 8, Essex, UK	2010	A	pos
NL45	onion	field	Field 8, Essex, UK	2010	A	pos
NL61	onion	field	Field 8, Essex, UK	2010	A	pos
NL66	onion	field	Field 8, Essex, UK	2010	A	pos
NL68	onion	field	Field 8, Essex, UK	2010	A	pos
NL70/1	onion	field	Field 8, Essex, UK	2010	A	pos
NL70/7	onion	field	Field 8, Essex, UK	2010	A	pos
NL71	onion	field	Field 8, Essex, UK	2010	A	n.i.
NL96	onion	field	Field 8, Essex, UK	2010	A	neg
SM105	onion	field	Field 7, Essex, UK	2010	I	neg
SM107	onion	field	Field 7, Essex, UK	2010	A	pos
SM112	onion	field	Field 7, Essex, UK	2010	A	pos
SM113	onion	field	Field 7, Essex, UK	2010	A	pos
SM114_2	onion	field	Field 7, Essex, UK	2010	A	n.i.
SM115a	onion	field	Field 7, Essex, UK	2010	A	pos
SM115b	onion	field	Field 7, Essex, UK	2010	A	pos
SM116a	onion	field	Field 7, Essex, UK	2010	A	pos
SM116b	onion	field	Field 7, Essex, UK	2010	A	pos
SM117	onion	field	Field 7, Essex, UK	2010	A	pos
SM118	onion	field	Field 7, Essex, UK	2010	A	neg
SM120	onion	field	Field 7, Essex, UK	2010	A	pos
SM122	onion	field	Field 7, Essex, UK	2010	A	pos
SM123	onion	field	Field 7, Essex, UK	2010	A	pos

SM14	onion	field	Field 7, Essex, UK	2010	A	pos
SM16	onion	field	Field 7, Essex, UK	2010	A	pos
SM17	onion	field	Field 7, Essex, UK	2010	A	pos
SM19	onion	field	Field 7, Essex, UK	2010	A	pos
SM2	onion	field	Field 7, Essex, UK	2010	A	n.i.
SM21	onion	field	Field 7, Essex, UK	2010	A	pos
SM23	onion	field	Field 7, Essex, UK	2010	A	n.i.
SM24	onion	field	Field 7, Essex, UK	2010	A	pos
SM27	onion	field	Field 7, Essex, UK	2010	A	pos
SM3	onion	field	Field 7, Essex, UK	2010	A	pos
SM30	onion	field	Field 7, Essex, UK	2010	A	neg
SM35	onion	field	Field 7, Essex, UK	2010	A	pos
SM36	onion	field	Field 7, Essex, UK	2010	A	pos
SM37	onion	field	Field 7, Essex, UK	2010	A	pos
SM4	onion	field	Field 7, Essex, UK	2010	A	pos
SM42	onion	field	Field 7, Essex, UK	2010	A	pos
SM47	onion	field	Field 7, Essex, UK	2010	A	pos
SM51	onion	field	Field 7, Essex, UK	2010	A	pos
SM54	onion	field	Field 7, Essex, UK	2010	A	neg
SM57	onion	field	Field 7, Essex, UK	2010	A	pos
SM58?	onion	field	Field 7, Essex, UK	2010	A	pos
SM68	onion	field	Field 7, Essex, UK	2010	A	n.i.
SM70	onion	field	Field 7, Essex, UK	2010	A	pos
SM74	onion	field	Field 7, Essex, UK	2010	A	pos
SM75	onion	field	Field 7, Essex, UK	2010	A	pos
SM75b	onion	field	Field 7, Essex, UK	2010	A	pos
SM76	onion	field	Field 7, Essex, UK	2010	A	pos
SM88	onion	field	Field 7, Essex, UK	2010	A	pos
SM88_2	onion	field	Field 7, Essex, UK	2010	A	pos
SM9	onion	field	Field 7, Essex, UK	2010	A	pos
SM90	onion	field	Field 7, Essex, UK	2010	I	neg
SM94	onion	field	Field 7, Essex, UK	2010	A	pos
SM99	onion	field	Field 7, Essex, UK	2010	A	pos
A1/2	onion	stored	Field 17, Warwickshire	2008	B	neg
A3/1	onion	stored	Field 17, Warwickshire	2008	A	pos
A3/2	onion	stored	Field 17, Warwickshire	2008	A	pos
Fus 2	onion	n.i.	Lincolnshire, UK	n.i.	A	pos
R11	onion	field	Field 14, Lincolnshire	2009	B	neg
RO2	onion	stored	Field 15, Lincolnshire, UK	2010	A	pos
Fus 1	onion	n.i.	Nottinghamshire, UK	n.i.	C	neg
Fus 3	onion	n.i.	Nottinghamshire, UK	n.i.	A	pos
A5	onion	stored	Field 16, Nottinghamshire	2009	C	neg
PG	onion	n.i.	Cambridgeshire, UK	n.i.	D	neg
FOB	onion	n.i.	Ireland	n.i.	A	pos
25	onion	n.i.	USA	1999	A	pos
NRRL 22538	onion	n.i.	Germany	n.i.	E	neg
151	onion	n.i.	Netherlands	2003	D	neg
180	onion	n.i.	Netherlands	2003	B	neg
181	onion	n.i.	Netherlands	n.i.	A	pos
260	onion	n.i.	Netherlands	2002	A	pos
262	onion	n.i.	Netherlands	2002	A	pos
739	onion	n.i.	Netherlands	2006	A	pos

D1	onion	set	Netherlands	2009	A	pos
D2	onion	set	Netherlands	2009	B	neg
D5	onion	set	Netherlands	2009	A	pos
D7/2/b	onion	set	Netherlands	2009	F	neg
H2	onion	stored	Netherlands	2010	A	pos
H4	onion	stored	Netherlands	2010	A	pos
H6	onion	stored	Netherlands	2010	A	neg
H7	onion	stored	Netherlands	2010	A	pos
B12	onion	set	Belgium	2009	B	neg
Sp2	onion	stored	Spain	2010	A	pos
Sp5_1	onion	stored	Spain	2010	B	neg
Sp7_2	onion	stored	Spain	2010	B	neg
Sp8	onion	stored	Spain	2010	A	pos
Sp11	onion	stored	Spain	2010	B	neg
Sp14	onion	stored	Spain	2010	B	neg
Sp16	onion	stored	Spain	2010	K	pos
Sp17	onion	stored	Spain	2010	A	pos
Sp18	onion	stored	Spain	2010	B	neg
CH1-1	onion	stored	Chile	2010	A	pos
CH1-2	onion	stored	Chile	2010	A	pos
CH1-3	onion	stored	Chile	2010	A	pos
CH3-1	onion	stored	Chile	2010	A	pos
CH5-1	onion	stored	Chile	2010	B	neg
CH4-2	onion	stored	Chile	2010	A	pos
CH5-2	onion	stored	Chile	2010	A	neg

n.i.: no information

APPENDIX III

Table 36. Host, origin, year of collection and *TEF* sequence type (ST) of *Fusarium* isolates associated with *Allium* spp. showing basal rot symptoms.

Species	Name	Collected	Origin	Year	TEF
F. proliferatum	FOA	onion	Ireland	n.i.	FP1
	A2/2	onion	Field 1, Warwickshire, UK	2008	FP2
	A5/1	onion	Field 1, Warwickshire, UK	2008	FP1
	A6/1	onion	Field 1, Warwickshire, UK	2008	FP2
	R9	onion	Field 14, Lincolnshire, UK	2009	FP1
	R16	onion	Field 14, Lincolnshire, UK	2009	FP1
	A40	onion	Field 4, Bedfordshire, UK	2009	FP1
	A8	onion	Field 4, Bedfordshire, UK	2009	FP2
	GR1	garlic	Bedfordshire, UK	2010	FP4
	Sp1_2	onion	Spain	2010	FP3
	Sp5_2	onion	Spain	2010	FP3
	Sp6	onion	Spain	2010	FP1
	Sp10_2	onion	Spain	2010	FP2
	Sp12	onion	Spain	2010	FP3
	31	onion	USA	1999	FP1
	50	onion	USA	1999	FP1
	CH2-1	onion	Chile	2010	FP1
F. avenaceum	C1	onion	Ireland	n.i.	FA5
	R2	onion	Field 10, Suffolk, UK	2009	FA4
	Fre2	onion	France	2009	FA3
	LB8	leek	Cambridgeshire, UK	2010	FA1
	LB9	leek	Cambridgeshire, UK	2010	FA1
	Sp9	onion	Spain	2010	FA2
F. solani	P3B	onion	Field 5, Essex, UK	2009	FS6
	R4	onion	Field 10, Suffolk, UK	2009	FS5
	R12	onion	Field 14, Lincolnshire, UK	2009	FS6
	G9	onion	Field 12, Suffolk, UK	2009	FS1
	G10	onion	Field 12, Suffolk, UK	2009	FS3
	A30	onion	Field 4, Bedfordshire, UK	2009	FS6
	A9	onion	Field 4, Bedfordshire, UK	2009	FS7
	A2	onion	Field 5, Essex, UK	2009	FS2
	A70	soil	Field 5, Essex, UK	2009	FS1
	A42	soil	Field 5, Essex, UK	2009	FS1
	A55	soil	Field 5, Essex, UK	2009	FS1
	I1	soil	Field 11, Suffolk, UK	2009	FS1
	Gr5-4	garlic	Bedfordshire, UK	2010	FS4
F. equiseti	A66	soil	Field 10, Suffolk, UK	2009	FE
	A73	soil	Field 10, Suffolk, UK	2009	FE
	A78	soil	Field 10, Suffolk, UK	2009	FE
F. acuminatum	Sp7_1	onion	Spain	2010	Fav

APPENDIX III (Continued)

F. redolens	SM4_2	onion	Field 7, Essex, UK	2010	FR1
	SM71	onion	Field 7, Essex, UK	2010	FR1
	NL41	onion	Field 8, Essex, UK	2010	FR2
	NL58	onion	Field 8, Essex, UK	2010	FR1
	NL69_1	onion	Field 8, Essex, UK	2010	FR1
	A683	soil	Field 5, Essex, UK	2009	FR1
	A77	soil	Field 5, Essex, UK	2009	FR1
	A76	soil	Field 5, Essex, UK	2009	FR1
	A74	soil	Field 5, Essex, UK	2009	FR1
	K3a	onion	Field 12, Suffolk, UK	2009	FR3
	G7	onion	Field 11, Suffolk, UK	2009	FR4
	G8	onion	Field 11, Suffolk, UK	2009	FR5
	G11	onion	Field 11, Suffolk, UK	2009	FR6
	PR2-1	onion	Field 5, Essex, UK	2009	FR1
F. culmorum	G47	soil	Field 12, Suffolk, UK	2009	FC
	A91	soil	Field 5, Essex, UK	2009	FC
	LLI1	leek	Cambridgeshire, UK	2010	FC
	LLI6	leek	Cambridgeshire, UK	2010	FC
	LNI2	leek	Cambridgeshire, UK	2010	FC
	LN2	leek	Cambridgeshire, UK	2010	FC
	LN5	leek	Cambridgeshire, UK	2010	FC
	LB3	leek	Cambridgeshire, UK	2010	FC

n.i.: no information

APPENDIX IV

Table 37 Survival index of Hystar F1 onion variety (black letters) and Striker F1 leek variety (red letters) seedlings when inoculated with 64 different *Fusarium* isolates from January to June in 2012 (angular transformed data, REML analysis, general linear model, LSD 5% level).

Batch/ Bench	Treatments															L.S.D.5 %	F pr
I/1.	Fus2	180	Fus1	22544	PG	22538	151	22	A28	18B	A19	Fom004	PR5	FOA4	Untrea		
	11.38	21.15	24.78	26.3	30.68	31.06	31.22	31.71	31.84	32.12	33.12	33.76	37.56	38.79	53.11	9.705	<0.001
I/2	Fus2	NL70_7	D2	SP7_2	A1/2	PR7	SH1_1	25	Gr4	M9	A5	SM105	FOA5	A13*	Untrea		
	10.22	11.38	12.2	13.49	14.35	17.7	18.53	18.7	21.15	26.69	32.03	37.22	38.8	54.99	54.41	10.05	<0.001
II/1	Fus2 *	Fus3	A35	262	A1/2	Gr5_1	FOB	26990	36311	Fo47	26988	26993	36425	26222	Untrea		
	63.16	25.59	28.63	29.19	30.68	35.2	35.59	37.1	38.15	40.84	45.01	47.64	48.08	50.32	63.4	9.144	<0.001
II/2	Fus2	SP2	A14	F1	SM54	H2	NL34	SIB	CH1_1	SH3	SH4	RO2	GR2	SP14	Untrea		
	16.47	12.28	17.83	20.45	20.89	22.83	24.43	25.13	25.2	27.97	28.03	35.3	37.07	43.45	56.28	9.695	<0.001
III/1	Fus2	180	D2	A13	36425	26988	Fus1	36311	A5	FOA5	Fom004	Fo47	NL96*	26222	Untrea		
	11.7	23.3	25.46	33.92	40.87	41.87	41.91	42.42	43.94	44.48	44.49	45	45.05	47.72	62.3	8.354	<0.001
IV/1	Fus2	22544	SM54	SP7_2	PR7	NL70_ 7	18B	18	PG	PR5	Fo47	SM105	FOA4	22538	Untrea		
	5.45	6.60	7.75	7.75	8.17	8.65	15.17	18.98	23.37	29.91	30.68	31.40	31.68	39.17	58.59	8.664	<0.001
IV/2	Fus2	A8	SP1-2	A40	SP6	SP10-2	A6/1	R9	50	CH2-1	R16	FOA	31	NL69	Untrea		
	9.32	6.6	11.7	12.53	14.1	14.9	17.05	17.18	17.3	17.62	18.78	21.5	22.57	25.64	50.23	9.399	<0.001
V/1	Fus2	18B	PG	FOA	NL69	TH2	22	Fo47	K3a	NL41	FOLR1	NL58	NL96	22538	Untre		
	7.56	37.17	34.33	38.1	38.72	40.92	42	42.88	44.82	45.78	47.08	47.12	49.99	56.93	59.42	11.32	<0.001
V/2	Fus2	SP7_1	A8	A40	18	31	Gr5-2	G10	A9	C1	Untrea	Fus2_lee k	Untreat ed-leek	LB9_leek	18B_leek		
	16.72	19.15	21.44	25.19	31.73	40.29	44.28	47.08	51.41	59.01	60.31	50.28	52.44	53.95	56.5	10.96	<0.001

Continuing

Appendix IV (Continued)

Date	Treatments															L.S.D.5 %	F pr
III/2.	Fus2-50x	180-50x	D2-50x	NL96-50x	A13-50x	Fus1-50x	FOA5-50x	36425-50x	26988-50x	26222-50x	Fo47-50x	A5- 50x	36311-50x	Fom004-50x	Untrea		
	24.55	41.71	42.4	49.71	50	50.21	50.81	52.24	52.86	53.84	53.85	55.52	57.91	58.27	60.12	8.806	<0.001

Survival index: Percentage of surviving onion seedlings (angular transformed data)
Isolates are considered very aggressive when they are not significantly different from Fus2 treatment (positive control)
Isolates are considered non-aggressive when they are not significantly different from Fo47 treatment (negative control)
Isolates are considered aggressive when significantly different from Fo47 treatment (negative control)
Isolates are considered non-aggressive as not significantly different from Untreated (water immersed)
*: experimental error, non-inoculated, data ignored
50x: spore concentration 50x diluted compare to other treatments ($2*10^4$ CFU/ml)

Appendix IV(Continued)

Table 38. Combined dataset of survival index of Hystar F1 onion seedlings when inoculated with 64 different *Fusarium* isolates. REML analysis, general linear model, LSD 5% level. Overall maximum L.S.D. 5%: 11.393.

Treatment	No. of repeates	Predicted mean	L.S.D.5%_ Fus2	L.S.D.5%_ Fo47	L.S.D.5%_ Untreated
SP2	1	10.44	8.406	9.081	8.385
Fus2	8	12.13	*	4.549	3.427
SP7_1	1	14.09	8.131	8.807	8.107
A14	1	15.99	8.406	9.081	8.385
A8	2	16.13	6.044	6.903	6.009
NL70_7	2	16.19	5.846	6.426	5.776
SP7_2	2	16.79	5.846	6.426	5.776
SM54	2	17.73	5.934	6.655	5.889
D2	2	18.19	5.889	6.386	5.791
F1	1	18.62	8.406	9.081	8.385
PR7	2	19.11	5.846	6.426	5.776
SP1-2	1	19.62	8.051	8.695	8.024
Fus3	1	19.67	8.286	8.313	8.071
H2	1	20.99	8.406	9.081	8.385
180	2	21.16	5.839	6.338	5.75
A40	2	21.27	6.044	6.903	6.009
A1/2	2	21.41	6.048	6.383	5.861
SP6	1	22.02	8.051	8.695	8.024
22544	2	22.17	5.823	6.403	5.763
SH1_1	1	22.23	7.944	8.426	7.886
25	1	22.4	7.944	8.426	7.886
NL34	1	22.59	8.406	9.081	8.385
A35	1	22.72	8.286	8.313	8.071
SP10-2	1	22.82	8.051	8.695	8.024
262	1	23.27	8.286	8.313	8.071
SIB	1	23.29	8.406	9.081	8.385
CH1_1	1	23.36	8.406	9.081	8.385
Gr4	1	24.85	7.944	8.426	7.886
A6/1	1	24.97	8.051	8.695	8.024
R9	1	25.1	8.051	8.695	8.024
50	1	25.22	8.051	8.695	8.024
CH2-1	1	25.54	8.051	8.695	8.024
SH3	1	26.14	8.406	9.081	8.385
SH4	1	26.19	8.406	9.081	8.385
R16	1	26.7	8.051	8.695	8.024
18	2	28.38	5.832	6.551	5.785
A13	1	28.98	7.873	8.143	7.786
Gr5_1	1	29.28	8.286	8.313	8.071
FOB	1	29.67	8.286	8.313	8.071

M9	1	30.39	7.944	8.426	7.886
18B	3	30.54	4.975	5.599	4.906
26990	1	31.18	8.286	8.313	8.071
FOA	2	31.61	5.882	6.542	5.835
PG	3	31.84	4.975	5.599	4.906
Fus1	2	32.27	5.839	6.338	5.75
31	2	33	6.044	6.903	6.009
RO2	1	33.47	8.406	9.081	8.385
151	1	34.02	7.855	8.338	7.811
A28	1	34.64	7.855	8.338	7.811
NL69	2	34.82	5.562	6.207	5.502
36311	2	34.86	6.105	6.298	5.903
GR2	1	35.23	8.406	9.081	8.385
A19	1	35.91	7.855	8.338	7.811
22	2	36.07	5.85	6.398	5.792
TH2	1	36.62	7.895	8.242	7.853
A5	2	37.37	5.889	6.386	5.791
NL96	1	37.92	6.421	6.863	6.337
26988	2	38.01	6.105	6.298	5.903
Fom004	2	38.05	5.839	6.338	5.75
Fo47	4	38.25	4.549	*	4.374
36425	2	39.05	6.105	6.298	5.903
PR5	2	39.45	5.823	6.403	5.763
SM105	2	40.48	5.846	6.426	5.776
K3a	1	40.59	7.895	8.242	7.853
Gr5-2	1	40.64	8.131	8.807	8.107
FOA4	2	40.96	5.823	6.403	5.763
FOA5	2	41.02	5.889	6.386	5.791
G10	1	41.06	8.131	8.807	8.107
NL41	1	41.3	7.895	8.242	7.853
SP14	1	41.62	8.406	9.081	8.385
26993	1	41.73	8.286	8.313	8.071
NL58	1	42.84	7.895	8.242	7.853
FOLR1	1	42.89	7.895	8.242	7.853
26222	2	43.59	6.105	6.298	5.903
22538	3	44.88	4.975	5.599	4.906
Fus2_leek	1	47.66	8.131	8.807	8.107
Untreated-leek	1	48.08	8.131	8.807	8.107
A9	1	48.53	8.131	8.807	8.107
LB9_leek	1	49.17	8.131	8.807	8.107
18B_leek	1	50.41	8.131	8.807	8.107
C1	1	55.29	8.131	8.807	8.107
Untreated	11	57.54	3.427	4.374	*

l.s.d. 5% Fus2: approximate least significant differences (5% level) of REML mens compared to Fus2 treatment

l.s.d. 5% Fo47:approximate least significant differences (5% level) of REML mens compared to Fo47 treatment

REML variance components analysis

Response variate:	ANGULARSurvival
Fixed model:	Constant + Treatment
Random model:	Date + Date.Row + Date.Row.Column
Number of units:	533

Estimated variance components

Random term	component	s.e.
Date	31.5	16.55
Date.Row	2.88	1.74
Date.Row.Column	39.95	35

Residual variance model

Term	Factor	Model(order)	Parameter	Estimate	s.e.
Residual		Identity	Sigma2	11.43	34.76

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	1382.88	81	16.61	295.6	<0.001

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	1382.88	81	16.61	295.6	<0.001

Standard error: 1.861

APPENDIX V

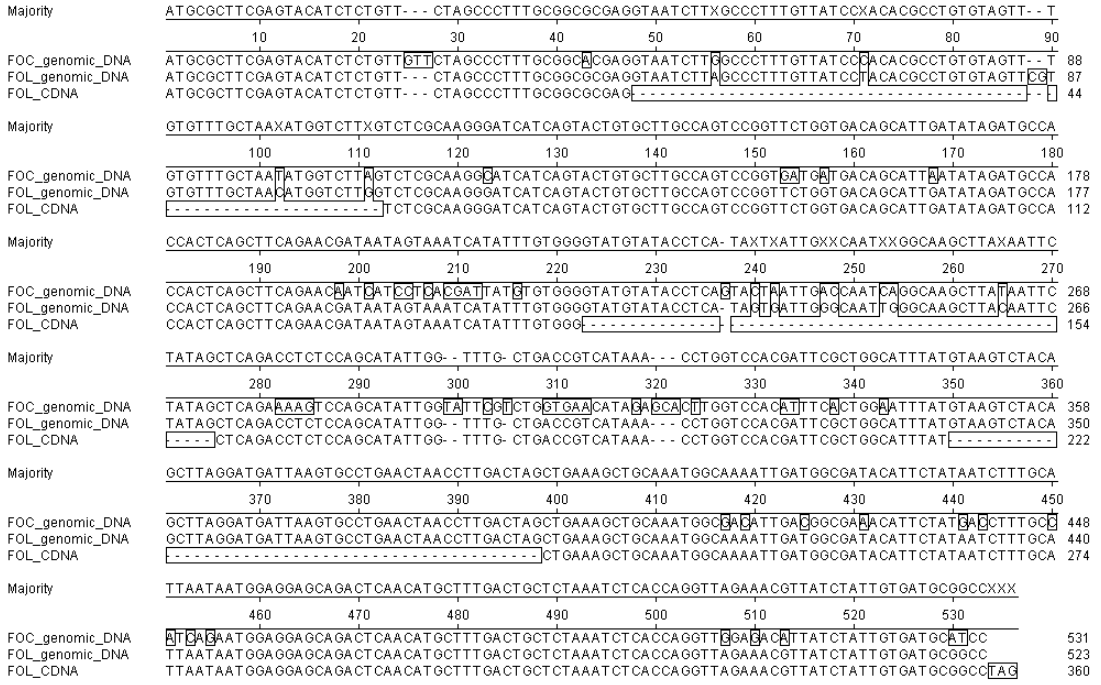
Table 39. Fragment sizes of 40 *Fusarium oxysporum* isolates using six microsatellite markers.

	FOL680	MB2	FOL35	FOL245	FOL356	21384
22	193.1	252.1	214.8	211.1	305.2	185.9
25	198.7	259.8	231.2	135.4	242.2	187.4
151	200.4	259.8	222.1	135.3	218	121.7
180	201.4	259.7	231	221.6	224	n.i.
262	198.8	259.6	231.3	135.5	242.2	187.2
18B	203.8	259.8	231.3	135.5	239.2	137.6
A1/2	201.7	259.5	224.2	222.1	224.1	119.3
A13	196.2	247.8	218	233.9	202.8	65.2
A14	203.9	250.9	222.3	135.6	248.2	187.3
A19	203.8	251.9	222	135.4	248.2	187.3
A28	199.5	259.5	230.3	135.5	299.4	N.D.
A35	198.6	259.8	231.1	135.3	242	187.3
A5	197.4	273.5	222.3	135.4	202.9	N.D.
D2	201.5	259.5	230.3	221.8	224.1	N.D.
FO47	199	256	255	214.6	120.1	120
FOA4	199.5	259.3	231.3	231.3	317.5	N.D.
FOA5	194.1	265.9	218.8	240.1	212	125.8
FOB	198.7	259.6	231.2	135.4	242	187.2
Fom004	203.6	251.7	225.2	135.4	248.2	137.7
Fus1	197.4	273.7	222.2	135.3	202.9	125.8
Fus2	198.5	259.6	230	135.3	242	187.2
Fus3	198.7	259.6	230.5	135.5	242.1	187.2
Gr4	198.7	259.8	231.2	135.4	242.1	N.D.
M9	195.3	247	218.3	267.6	202.9	118.2
N70_7	204	259.9	221.4	135.6	248.2	118.2
NRRL22538	198.3	242.4	234.2	141.9	208.8	N.D.
NRRL22544	198.8	259.9	231.3	135.5	242.2	187.4
NRRL26222	199.4	241.7	211.8	255.7	202.7	291.4
NRRL26988	199.4	241.4	213.1	224.4	202.7	181.8
NRRL26990	199.4	259.5	231.1	217.5	326.4	137.6
NRRL26993	197.4	273.7	222	135.2	202.9	125.7

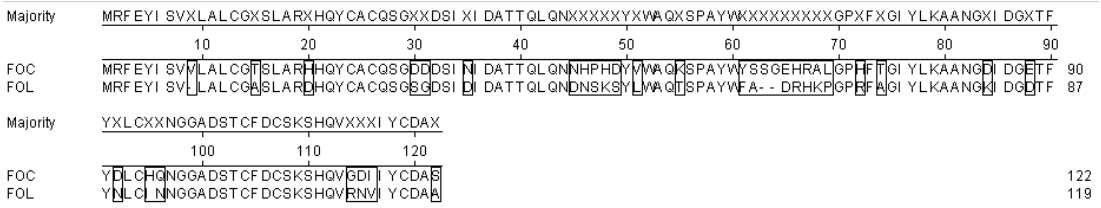
NRRL36311	203.8	261.5	231.1	135.5	299.4	N.D.
NRRL36425	203.7	259.9	231.1	135.4	286	148.5
PG	201.6	251.5	222.1	135.5	267.1	n.i.
PR5	198.7	249.8	237.2	135.5	308.4	N.D.
PR7	201.5	259.5	224.2	221.9	224	n.i.
SH1_1	200.7	259.7	222.5	135.6	218.1	120.2
SIB	203.9	251.8	221.2	135.6	248.2	187.2
SM105	196.4	247	218.3	231.1	202.9	119.7
SM54	199.4	259.8	230.2	217.6	267.1	137.6

APPENDIX VI

Figure 36. A–D Nucleotide and amino acid alignment of *SIX5* and *SIX9* of *Fusarium oxysporum* formae speciales *cepae* and *lycopersici.*



A Nucleotide sequence alignment of genomic and coding region of *SIX5* of *F. oxysporum* f. sp. *lycopersici* and homologue of *SIX5* of *F. oxysporum* f. sp. *cepae*

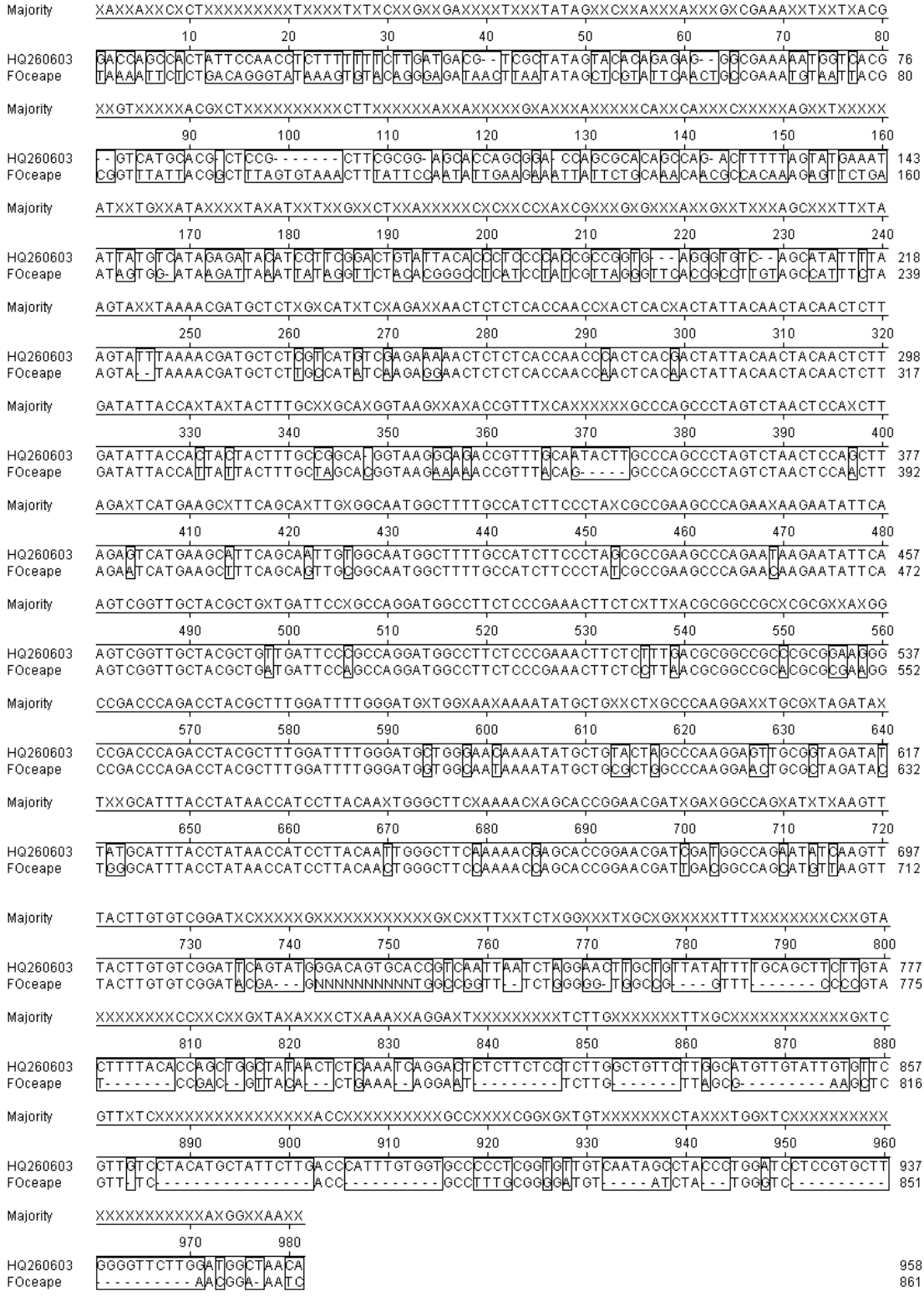


B Amino acid sequence alignment of SIX5 of *F. oxysporum* f. sp. *lycopersici* and homologue of SIX5 of *F. oxysporum* f. sp. *cepae*

```
FOC          399  MKLSAVAAMAFAIFPIAEAQNKNIQVGCYADDSSQDGllpkllnaaarakadpdlrFGF  578
                  MK SA+ AMAFAIFP AEAQNKNIQVGCYA DS QDGLLPKLL +AAAR +ADPDLRFGF
EGU79523.    1    MKHSAIVAMAFAIFPSAEAQNKNIQVGCYAVDSRQDGLLPKLLFDAAARGRADPDLRFGF  60

FOC          579  WDGGNKICCAGPRNCARYWAFTYNHPYNWASKTSTGTIDGQHVKFTCVGYE  731
                  WD GNKICC  PR+C RY+AFTYNHPYNWASKTSTGTIDGQ++KFTCVG+
EGU79523     61   WDAGNKICCTSPRSCGRYYAFTYNHPYNWASKTSTGTIDGQNIKFTCVGFS  111
```

C Amino acid sequence alignment of SIX9 of *F. oxysporum* f. sp. *lycopersici* and homologue of SIX9 of *F. oxysporum* f. sp. *cepae*



D Nucleotide sequence alignment of *SIX9* of *F. oxysporum* f. sp. *lycopersici* and homologue of *SIX9* of *F. oxysporum* f. sp. *cepae*

APPENDIX VII

Table 40. Sequence description, length, similarity to closest hit of 600 (509 excluding alternatively spliced) predicted proteins of *Fusarium oxysporum* f. sp. *cepae* which do not present in *F. oxysporum* f. sp. *lycopersici* 4287.

Predicted protein	Sequence description	Sequence length	Hit ACC	Similarity
Contig_2_consensus_sequence:g1.t1	glutathione s-	726	EGU83821	99
Contig_2_consensus_sequence:g2.t1	phenol 2-	1881	EGU75771	98
Contig_2_consensus_sequence:g2.t2	phenol 2-	1869	EGU75771	98
Contig_2_consensus_sequence:g3.t1	c6 transcription	1800	EGU75772	88
Contig_2_consensus_sequence:g4.t1	c6 zinc finger domain protein	1254	EGU75773	99
Contig_2_consensus_sequence:g4.t2	c6 zinc finger domain protein	1389	EGU75773	98
Contig_2_consensus_sequence:g5.t1	hypothetical protein FOXB_13687 [Fusarium oxysporum Fo5176]	267	EGU75775	100
Contig_2_consensus_sequence:g6.t1	hypothetical protein FOXB_13688 [Fusarium oxysporum Fo5176]	1089	EGU75776	82
Contig_2_consensus_sequence:g7.t1	f-box domain protein	858	EGU75776	96
Contig_2_consensus_sequence:g8.t1	small secreted protein	375	EGU75777	100
Contig_2_consensus_sequence:g9.t1	polyketide synthase	1179	AAS57287	78
Contig_2_consensus_sequence:g9.t2	polyketide synthase	1155	AAS57287	79
Contig_2_consensus_sequence:g10.t1	hypothetical protein FG04693.1 [Gibberella zeae PH-1]	1209	XP_384869	96
Contig_2_consensus_sequence:g11.t1	acyl transferase	1512	EGU75781	98
Contig_2_consensus_sequence:g12.t1	udp- transferase	2550	EGU75782	98
Contig_2_consensus_sequence:g12.t2	udp- transferase	2991	EGU75782	97
Contig_2_consensus_sequence:g12.t3	hypothetical protein FG04691.1 [Gibberella zeae PH-1]	525	XP_384867	96
Contig_2_consensus_sequence:g13.t1	hypothetical protein FOXB_13695 [Fusarium oxysporum Fo5176]	489	EGU75783	98
Contig_2_consensus_sequence:g14.t1	secreted protein	3015	XP_391251	90
Contig_2_consensus_sequence:g14.t2	secreted protein	3462	XP_391251	90
Contig_2_consensus_sequence:g15.t1	phenylacetaldoxime dehydratase	2934	EGU75787	88
Contig_2_consensus_sequence:g16.t1	and nb-arc domain-containing protein	5643	XP_003050547	58
Contig_2_consensus_sequence:g17.t1	hypothetical protein FOXB_13700 [Fusarium oxysporum Fo5176]	2643	EGU75788	83
Contig_2_consensus_sequence:g17.t2	hypothetical protein FOXB_13700 [Fusarium oxysporum Fo5176]	2253	EGU75788	88
Contig_2_consensus_sequence:g18.t1	family protein	1080	EGU75789	98
Contig_2_consensus_sequence:g19.t1	glycosyl family 2	3015	EGU75790	98
Contig_2_consensus_sequence:g20.t1	trichothecene 3-o-acetyltransferase	1365	ADQ52719	99
Contig_2_consensus_sequence:g21.t1	protein ssxt	1839	EGU75791	98
Contig_2_consensus_sequence:g22.t1	pfs domain protein	3201	EGU75793	93
Contig_2_consensus_sequence:g23.t1	c6 transcription factor	1902	EGU75794	91

Contig_2_consensus_sequence:g23.t2	c6 transcription factor	1971	EGU75794	95
Contig_2_consensus_sequence:g24.t1	leucyl aminopeptidase (aminopeptidase t)-like protein	1113	EGU75794	96
Contig_2_consensus_sequence:g25.t1	mfs monocarboxylate	696	EGU75795	98
Contig_2_consensus_sequence:g25.t2	mfs monocarboxylate	702	EGU75795	100
Contig_2_consensus_sequence:g26.t1	hypothetical protein FOXB_13708 [Fusarium oxysporum Fo5176]	954	EGU75796	98
Contig_2_consensus_sequence:g26.t2	hypothetical protein FOXB_13708 [Fusarium oxysporum Fo5176]	1008	EGU75796	98
Contig_2_consensus_sequence:g27.t1	carbonic anhydrase	1749	EGU75799	96
Contig_2_consensus_sequence:g27.t2	carbonic anhydrase	1701	EGU75799	96
Contig_2_consensus_sequence:g28.t1	prolyl oligopeptidase	1098	EGU75800	98
Contig_2_consensus_sequence:g29.t1	hypothetical protein FOXB_13713 [Fusarium oxysporum Fo5176]	537	EGU75801	92
Contig_2_consensus_sequence:g30.t1	c6 finger domain	1218	XP_385027	82
Contig_5_consensus_sequence:g31.t1	hypothetical protein NECHADRAFT_75597 [Nectria haematococca mpVI 77-13-4]	1875	XP_003053942	70
Contig_5_consensus_sequence:g31.t2	mhc_i c-terminus family protein	2217	XP_003053942	69
Contig_5_consensus_sequence:g32.t1	2-nitropropane dioxygenase	1059	EGU75078	98
Contig_5_consensus_sequence:g33.t1	tpr domain protein	3048	EGU75079	93
Contig_5_consensus_sequence:g34.t1	glycoside hydrolase family 115 protein	3192	EGU75080	99
Contig_5_consensus_sequence:g35.t1	het-domain-containing protein	2358	XP_383513	65
Contig_5_consensus_sequence:g36.t1	purine transporter	1614	XP_003045683	93
Contig_5_consensus_sequence:g36.t2	purine transporter	1761	XP_003045683	93
Contig_5_consensus_sequence:g37.t1	sh3 domain-containing protein	2391	XP_003045555	74
Contig_5_consensus_sequence:g37.t2	sh3 domain-containing protein	2301	XP_003045555	74
Contig_5_consensus_sequence:g38.t1	abc multidrug	4404	EGU75083	99
Contig_5_consensus_sequence:g39.t1	tri15 protein	969	EGU75084	99
Contig_5_consensus_sequence:g40.t1	s-adenosyl-l-methionine-dependent methyltransferase	1377	EGU75085	99
Contig_5_consensus_sequence:g41.t1	integral membrane protein	1137	EGU75086	100
Contig_5_consensus_sequence:g41.t2	integral membrane protein	1089	EGU75086	95
Contig_5_consensus_sequence:g42.t1	hypothetical protein FOXB_14401 [Fusarium oxysporum Fo5176]	1920	EGU75087	96
Contig_5_consensus_sequence:g43.t1	phospholipase c	1929	EGU75089	95
Contig_5_consensus_sequence:g44.t1	mannosyl phosphorylinositol ceramide synthase sur1	1077	EGU75090	99
Contig_5_consensus_sequence:g45.t1	ankyrin repeat and sam domain containing protein 6	1731	EGU75091	97
Contig_6_consensus_sequence:g46.t1	gaba permease	1533	EGU84107	100
Contig_6_consensus_sequence:g47.t1	msf superfamily transporter	1635	EGU84108	99
Contig_6_consensus_sequence:g48.t1	c6 transcription factor	963	EGU84109	99
Contig_6_consensus_sequence:g49.t1	c6 transcription factor	855	EGU84109	100
Contig_6_consensus_sequence:g50.t1	alpha-galactosidase	3540	EGU84110	97
Contig_6_consensus_sequence:g50.t2	alpha-galactosidase c	3495	EGU84110	99
Contig_6_consensus_sequence:g51.t1	hypothetical protein FOXB_05359 [Fusarium oxysporum Fo5176]	480	EGU84112	94
Contig_6_consensus_sequence:g52.t1	polyketide cyclase dehydrase	456	XP_003001601	82

Contig_6_consensus_sequence:g53.t1	polyketide cyclase dehydrase	429	XP_003001600	79
Contig_6_consensus_sequence:g54.t1	c2h2 type zinc finger domain protein	1044	XP_003001599	75
Contig_6_consensus_sequence:g54.t2	c2h2 type zinc finger domain protein	927	XP_003001599	75
Contig_6_consensus_sequence:g55.t1	cytochrome p450 monooxygenase pc-bph	1500	EGU84114	91
Contig_6_consensus_sequence:g55.t2	cytochrome p450 benzoate 4-	1290	EGU84114	94
Contig_6_consensus_sequence:g56.t1	hypothetical protein FOXB_05362 [Fusarium oxysporum Fo5176]	681	EGU84115	98
Contig_6_consensus_sequence:g57.t1	aldehyde reductase ii	951	EGU84116	83
Contig_6_consensus_sequence:g58.t1	ethyl tert-butyl ether degradation	420	EGU84117	100
Contig_6_consensus_sequence:g59.t1	family protein	714	EGU84118	99
Contig_6_consensus_sequence:g60.t1	nadph-dependent methylglyoxal reductase gre2	1011	EGU84118	99
Contig_6_consensus_sequence:g61.t1	aflatoxin biosynthesis ketoreductase nor-1	750	EGU84119	97
Contig_6_consensus_sequence:g62.t1	short chain dehydrogenase	1908	EGU84121	91
Contig_6_consensus_sequence:g62.t2	short chain dehydrogenase	1614	EGU84121	96
Contig_6_consensus_sequence:g63.t1	phosphotransferase enzyme family protein	930	EGU84122	100
Contig_6_consensus_sequence:g64.t1	hypothetical protein FOXB_05370 [Fusarium oxysporum Fo5176]	624	EGU84123	99
Contig_6_consensus_sequence:g65.t1	saccharopine dehydrogenase	1188	EGU84124	88
Contig_6_consensus_sequence:g66.t1	ferric-chelate reductase - proteolysis	3228	EGU84125	98
Contig_6_consensus_sequence:g66.t2	ferric-chelate reductase - proteolysis	3201	EGU84125	98
Contig_7_consensus_sequence:g67.t1	hypothetical protein FOXB_15790 [Fusarium oxysporum Fo5176]	1068	EGU73700	48
Contig_7_consensus_sequence:g68.t1	ankyrin repeat protein	819	EGU77078	57
Contig_7_consensus_sequence:g69.t1	hypothetical protein MAC_05885 [Metarhizium acridum CQMa 102]	2079	EFY88021	45
Contig_7_consensus_sequence:g70.t1	liver stage	771	XP_001705674	44
Contig_7_consensus_sequence:g71.t1	hypothetical protein FOXB_12412 [Fusarium oxysporum Fo5176]	1011	EGU77074	62
Contig_7_consensus_sequence:g73.t1	ferric-chelate reductase	1785	EGU77070	47
Contig_7_consensus_sequence:g73.t2	fad-binding domain-containing protein	1569	EGU77070	49
Contig_7_consensus_sequence:g79.t1	domain-containing histone demethylation protein 3d	2517	EGU77112	50
Contig_8_consensus_sequence:g86.t1	hypothetical protein FOXB_05678 [Fusarium oxysporum Fo5176]	570	EGU83808	88
Contig_8_consensus_sequence:g87.t1	hypothetical protein FOXB_05679 [Fusarium oxysporum Fo5176]	1992	EGU83809	99
Contig_8_consensus_sequence:g88.t1	hypothetical protein FOXB_05680 [Fusarium oxysporum Fo5176]	798	EGU83810	98
Contig_8_consensus_sequence:g89.t1	subtilisin-like protease sub5	1749	EGU83811	97
Contig_8_consensus_sequence:g89.t2	subtilisin-like protease sub5	1725	EGU83811	98
Contig_8_consensus_sequence:g90.t1	amine flavin-containing	1824	EGU83812	99
Contig_8_consensus_sequence:g91.t1	hexose transporter protein	1566	EGU83813	99
Contig_8_consensus_sequence:g92.t1	agglutinin-like protein als2 fragment	6522	EGU83814	87
Contig_8_consensus_sequence:g93.t1	class iii chitinase 1	927	EGU83815	90
Contig_8_consensus_sequence:g94.t1	c6 transcription	1869	EGU83816	98
Contig_8_consensus_sequence:g95.t1	allantoate permease	1536	EGU83817	99
Contig_8_consensus_sequence:g96.t1	branched-chain amino acid	1029	EGU83818	99

Contig_8_consensus_sequence:g97.t1	alcohol dehydrogenase	1071	EGX88787	76
Contig_8_consensus_sequence:g97.t2	alcohol dehydrogenase	1083	EGX88787	76
Contig_8_consensus_sequence:g98.t1	von willebrand factor	642	EGU77538	75
Contig_8_consensus_sequence:g99.t1	serine threonine protein kinase	1521	XP_382589	62
Contig_8_consensus_sequence:g100.t1	hypothetical protein FG02414.1 [Gibberella zeae PH-1]	1776	XP_382590	68
Contig_8_consensus_sequence:g101.t1	chitin synthase a	831	EGU83820	93
Contig_8_consensus_sequence:g101.t2	chitin synthase a	759	EGU83820	94
Contig_12_consensus_sequence:g102.t1	ankyrin repeat protein	882	EGU86839	100
Contig_12_consensus_sequence:g103.t1	nuclear pore complex	1335	EGU77077	78
Contig_12_consensus_sequence:g104.t1	hypothetical protein FOXB_02645 [Fusarium oxysporum Fo5176]	297	EGU86842	98
Contig_12_consensus_sequence:g105.t1	hypothetical protein FOXB_02647 [Fusarium oxysporum Fo5176]	561	EGU86844	100
Contig_12_consensus_sequence:g106.t1	hypothetical protein FOXB_12422 [Fusarium oxysporum Fo5176]	3084	EGU77067	99
Contig_12_consensus_sequence:g107.t1	fad-binding domain-containing protein	1380	EGU73043	83
Contig_12_consensus_sequence:g108.t1	hypothetical protein FOXB_16446 [Fusarium oxysporum Fo5176]	492	EGU73042	100
Contig_12_consensus_sequence:g109.t1	hypothetical protein FOXB_16445 [Fusarium oxysporum Fo5176]	1599	EGU73041	93
Contig_12_consensus_sequence:g110.t1	hypothetical protein FOXB_16167 [Fusarium oxysporum Fo5176]	372	EGU73322	98
Contig_12_consensus_sequence:g111.t1	hypothetical protein FOXB_15641 [Fusarium oxysporum Fo5176]	1230	EGU73849	86
Contig_12_consensus_sequence:g112.t1	ankyrin repeat family protein	819	EGU86846	50
Contig_12_consensus_sequence:g113.t1	hypothetical protein FOXB_02637 [Fusarium oxysporum Fo5176]	876	EGU86848	85
Contig_12_consensus_sequence:g114.t1	ankyrin repeat protein	762	EGU86846	95
Contig_12_consensus_sequence:g116.t1	ion channel nompc	864	EGU86846	52
Contig_12_consensus_sequence:g117.t1	kinesin light chain	1332	XP_383253	69
Contig_13_consensus_sequence:g118.t1	carbonic anhydrase	3276	XP_001827320	45
Contig_13_consensus_sequence:g119.t1	hypothetical protein PMAA_095060 [Penicillium marneffei ATCC 18224]	915	XP_002149068	62
Contig_13_consensus_sequence:g120.t1	hypothetical protein PMAA_095060 [Penicillium marneffei ATCC 18224]	828	XP_002149068	54
Contig_13_consensus_sequence:g121.t1	carbon-nitrogen hydrolase	2892	XP_387380	85
Contig_13_consensus_sequence:g121.t2	carbon-nitrogen hydrolase	2916	XP_387380	86
Contig_13_consensus_sequence:g122.t1	dienelactone hydrolase family protein	741	EGU77555	99
Contig_13_consensus_sequence:g123.t1	c6 transcription	1989	EGU77554	77
Contig_13_consensus_sequence:g124.t1	duf1338 domain protein	1077	EGU77554	96
Contig_13_consensus_sequence:g125.t1	quinone oxidoreductase	987	EGU77553	97
Contig_13_consensus_sequence:g126.t1	para-nitrobenzyl esterase	1614	EGU77552	94
Contig_13_consensus_sequence:g127.t1	ring-cleavage extradiol dioxygenase	609	EGU77551	98
Contig_13_consensus_sequence:g128.t1	hypothetical protein FOXB_11940 [Fusarium oxysporum Fo5176]	940	EGU77550	98
Contig_16_consensus_sequence:g129.t1	hypothetical protein FOXB_05677 [Fusarium oxysporum Fo5176]	1482	EGU83807	98
Contig_16_consensus_sequence:g130.t1	nucleoside-diphosphate-sugar epimerase	2160	EGU83806	99
Contig_16_consensus_sequence:g130.t2	nucleoside-diphosphate-sugar epimerase	2106	EGU83806	99
Contig_16_consensus_sequence:g131.t1	nadph dehydrogenase	1107	EGU83805	98

Contig_16_consensus_sequence:g132.t1	fungal specific transcription	1707	EGU83804	98
Contig_16_consensus_sequence:g133.t1	deoxyribose-phosphate aldolase	792	EGU83804	99
Contig_16_consensus_sequence:g134.t1	outer membrane partial	1518	EGU83803	88
Contig_16_consensus_sequence:g135.t1	tpr domain containing protein	5685	EGU83803	98
Contig_16_consensus_sequence:g136.t1	alpha beta hydrolase	510	EGU83802	98
Contig_16_consensus_sequence:g137.t1	saccharopine dehydrogenase	1071	EGU83800	90
Contig_16_consensus_sequence:g138.t1	siderophore iron	1785	EGU83799	99
Contig_16_consensus_sequence:g138.t2	siderophore iron	1731	EGU83799	96
Contig_16_consensus_sequence:g139.t1	pathogenesis associated protein pep2	708	EGU83798	99
Contig_17_consensus_sequence:g140.t1	polysaccharide deacetylase family protein	603	EGU88717	98
Contig_17_consensus_sequence:g141.t1	metabolite transport	1455	EGU88718	95
Contig_17_consensus_sequence:g142.t1	alkaline phosphatase family protein	1614	EGU88719	89
Contig_17_consensus_sequence:g143.t1	protein	822	EGU88720	95
Contig_17_consensus_sequence:g144.t1	metal-activated pyridoxal enzyme	1101	EGU88721	99
Contig_17_consensus_sequence:g145.t1	mfs transporter	1473	EGU88721	99
Contig_17_consensus_sequence:g146.t1	esterase lipase	1008	EGU88722	100
Contig_17_consensus_sequence:g147.t1	class 3 chitinase 2	978	EGU88723	96
Contig_17_consensus_sequence:g148.t1	transcriptional activator srcap-like protein	5295	EGU86854	99
Contig_17_consensus_sequence:g149.t1	hypothetical protein FOXB_02617 [Fusarium oxysporum Fo5176]	3618	EGU86855	99
Contig_17_consensus_sequence:g150.t1	hypothetical protein FOXB_02618 [Fusarium oxysporum Fo5176]	4176	EGU86856	95
Contig_19_consensus_sequence:g151.t1	c6 finger domain	2241	EGU88738	98
Contig_19_consensus_sequence:g152.t1	glycerophosphoryl diester phosphodiesterase	807	EGU88739	99
Contig_19_consensus_sequence:g153.t1	myo-inositol-1(or 4)-monophosphatase	1059	XP_003051688	74
Contig_19_consensus_sequence:g154.t1	mfs maltose permease	1572	EGP87298	64
Contig_19_consensus_sequence:g154.t2	mfs maltose permease	1620	EGP87298	65
Contig_19_consensus_sequence:g155.t1	major facilitator superfamily transporter	1734	BAE66205	66
Contig_19_consensus_sequence:g156.t1	fad dependent oxidoreductase superfamily	1080	CBF71787	64
Contig_19_consensus_sequence:g157.t1	transcriptional activator protein acu-	1779	XP_003051041	61
Contig_19_consensus_sequence:g157.t2	transcriptional activator protein acu-	1632	XP_003051041	58
Contig_19_consensus_sequence:g158.t1	hypothetical protein NECHADRAFT_45174 [Nectria haematococca mpVI 77-13-4]	687	XP_003049125	81
Contig_19_consensus_sequence:g159.t1	dihydroxyacetone kinase	1758	XP_387193	79
Contig_19_consensus_sequence:g160.t1	aquaporin 3	987	EGR45687	81
Contig_19_consensus_sequence:g160.t2	aquaporin 3	930	EGR45687	75
Contig_19_consensus_sequence:g161.t1	c6 transcription factor	2133	EFQ34812	60
Contig_25_consensus_sequence:g162.t1	ga4 desaturase	391	EGU79214	96
Contig_25_consensus_sequence:g163.t1	alcohol dehydrogenase zinc-binding domain-containing protein	1014	XP_003041778	87
Contig_25_consensus_sequence:g164.t1	acetylglutamate	1083	XP_003051167	74
Contig_25_consensus_sequence:g165.t1	beta-glucosidase	2481	XP_003040768	86

Contig_25_consensus_sequence:g166.t1	rhamnosidase b	4011	XP_003040799	75
Contig_25_consensus_sequence:g167.t1	hexose transporter	1632	XP_003040798	91
Contig_25_consensus_sequence:g168.t1	short-chain dehydrogenases	891	EGU84376	89
Contig_25_consensus_sequence:g169.t1	aflatoxin biosynthesis ketoreductase nor-1	771	XP_003040805	82
Contig_25_consensus_sequence:g170.t1	n. i.	900	XP_003041839	81
Contig_25_consensus_sequence:g170.t2	n. i.	897	XP_003041839	81
Contig_25_consensus_sequence:g171.t1	n. i.	1008	EGU84473	75
Contig_25_consensus_sequence:g171.t2	n. i.	1299	EGU84473	75
Contig_22_consensus_sequence:g172.t1	carbonyl	999	XP_003050714	91
Contig_22_consensus_sequence:g172.t2	carbonyl	972	XP_003050714	91
Contig_22_consensus_sequence:g173.t1	l-arabinitol 4-dehydrogenase	1095	XP_003040137	82
Contig_22_consensus_sequence:g174.t1	nadph-cytochrome p450 reductase	2073	XP_750985	73
Contig_22_consensus_sequence:g175.t1	short chain dehydrogenase reductase family	828	XP_003042519	75
Contig_22_consensus_sequence:g176.t1	benzoate 4-monooxygenase cytochrome p450	1614	XP_001827680	54
Contig_22_consensus_sequence:g176.t2	benzoate 4-monooxygenase cytochrome p450	1560	XP_001827680	54
Contig_22_consensus_sequence:g177.t1	zn 2cys6 transcription factor	1590	CAK47054	49
Contig_22_consensus_sequence:g177.t2	zn 2cys6 transcription factor	1590	CAK47054	49
Contig_22_consensus_sequence:g178.t1	hexose carrier protein	1623	EDP51422	72
Contig_22_consensus_sequence:g178.t2	hexose carrier protein	1557	EDP51422	75
Contig_22_consensus_sequence:g179.t1	integral membrane protein	924	EGU87580	67
Contig_20_consensus_sequence:g180.t1	cytochrome p450 monooxigenase 2	3405	XP_001263169	89
Contig_20_consensus_sequence:g180.t2	cytochrome p450 monooxigenase 2	3288	XP_001263169	89
Contig_20_consensus_sequence:g181.t1	c6 transcription factor 2	885	XP_001263176	63
Contig_20_consensus_sequence:g182.t1	mfs transporter	1302	XP_001217046	89
Contig_20_consensus_sequence:g183.t1	o-methyltransferase	1317	XP_001263174	91
Contig_20_consensus_sequence:g184.t1	nonribosomal peptide synthase 2	7701	XP_001263173	84
Contig_20_consensus_sequence:g185.t1	c6 transcription	2208	XP_001263171	85
Contig_20_consensus_sequence:g185.t2	c6 transcription	2238	XP_001263171	83
Contig_23_consensus_sequence:g186.t1	nacht and tpr domain protein	4596	EGU72331	94
Contig_23_consensus_sequence:g186.t2	nacht and tpr domain protein	4551	EGU72331	94
Contig_23_consensus_sequence:g187.t1	c6 transcription	1452	EGU77533	88
Contig_23_consensus_sequence:g188.t1	lignostilbene dioxygenase family protein	1431	EGU77532	86
Contig_23_consensus_sequence:g189.t1	flavin-binding monooxygenase	1299	EGU77531	97
Contig_23_consensus_sequence:g190.t1	ascorbase and cu-oxidase	1764	EGU72208	99
Contig_23_consensus_sequence:g190.t2	ascorbase and cu-oxidase	1704	EGU72208	96
Contig_23_consensus_sequence:g191.t1	domain protein	5511	XP_003044128	82
Contig_23_consensus_sequence:g191.t2	domain protein	5331	XP_003044128	83
Contig_23_consensus_sequence:g192.t1	class v	1871	XP_001259036	67

Contig_24_consensus_sequence:g193.t1	transcriptional activator srcap-like protein	1142	EGX49225	72
Contig_24_consensus_sequence:g193.t2	transcriptional activator srcap-like protein	1136	EGX49225	72
Contig_24_consensus_sequence:g194.t1	hypothetical protein FOXB_05034 [Fusarium oxysporum Fo5176]	1092	EGU84441	97
Contig_24_consensus_sequence:g194.t2	hypothetical protein FOXB_05034 [Fusarium oxysporum Fo5176]	1086	EGU84441	96
Contig_24_consensus_sequence:g195.t1	related to light induced alcohol dehydrogenase bli-4	969	EGU84443	100
Contig_24_consensus_sequence:g196.t1	hypothetical protein FOXB_05038 [Fusarium oxysporum Fo5176]	609	EGU84445	98
Contig_24_consensus_sequence:g196.t2	hypothetical protein FOXB_05038 [Fusarium oxysporum Fo5176]	723	EGU84445	81
Contig_24_consensus_sequence:g197.t1	cysteine-rich repeat-containing protein	1596	EGU84446	99
Contig_24_consensus_sequence:g198.t1	heterokaryon incompatibility	1719	EGU84449	99
Contig_24_consensus_sequence:g199.t1	alkaline proteinase	1242	EGU84450	99
Contig_24_consensus_sequence:g200.t1	phthalate transporter	1608	EFY91116	81
Contig_24_consensus_sequence:g200.t2	phthalate transporter	1617	EFY91116	81
Contig_24_consensus_sequence:g201.t1	cytochrome p450 pisatin	1440	EGU84127	92
Contig_24_consensus_sequence:g201.t2	cytochrome p450 pisatin	1416	EGU84127	91
Contig_27_consensus_sequence:g202.t1	nad dependent epimerase	921	XP_003050218	73
Contig_27_consensus_sequence:g203.t1	para-nitrobenzyl esterase	1443	XP_003054728	73
Contig_27_consensus_sequence:g204.t1	fungal specific transcription factor	1737	XP_003044377	74
Contig_27_consensus_sequence:g205.t1	abc multidrug	1881	XP_003044376	87
Contig_27_consensus_sequence:g205.t2	abc multidrug	1902	XP_003044376	89
Contig_27_consensus_sequence:g206.t1	abc multidrug	2475	XP_003044376	91
Contig_27_consensus_sequence:g207.t1	oxidoreductase, putative [Aspergillus flavus NRRL3357]	381	XP_002379380	67
Contig_27_consensus_sequence:g208.t1	cytochrome p450	1485	XP_003048876	71
Contig_27_consensus_sequence:g209.t1	alpha beta hydrolase fold related protein	1023	EFY96989	73
Contig_29_consensus_sequence:g210.t1	metalloprotease mep1	447	XP_003050540	50
Contig_29_consensus_sequence:g211.t1	secreted family s9 peptidase	987	EGY16813	70
Contig_29_consensus_sequence:g212.t1	transcription factor cys6	1494	XP_003049506	64
Contig_29_consensus_sequence:g213.t1	protein	831	EFQ31450	65
Contig_29_consensus_sequence:g214.t1	salicylate 1-monooxygenase	930	XP_001215279	65
Contig_29_consensus_sequence:g215.t1	alcohol dehydrogenase	708	XP_001881024	55
Contig_29_consensus_sequence:g216.t1	aldehyde dehydrogenase	1047	XP_382568	64
Contig_29_consensus_sequence:g217.t1	c2h2 type zinc finger domain protein	2193	EGY16812	45
Contig_29_consensus_sequence:g218.t1	pyruvate decarboxylase	564	EGU88715	95
Contig_29_consensus_sequence:g219.t1	related to nuclear pore protein	795	EGU88714	97
Contig_28_consensus_sequence:g220.t1	hypothetical protein FG07824.1 [Gibberella zeae PH-1]	309	XP_388000	50
Contig_28_consensus_sequence:g221.t1	hypothetical protein CPC735_020520 [Coccidioides posadasii C735 delta SOWgp]	1122	XP_003065169	44
Contig_28_consensus_sequence:g221.t2	hypothetical protein FOXB_12996 [Fusarium oxysporum Fo5176]	1044	EGU76492	43
Contig_28_consensus_sequence:g222.t1	hypothetical protein CHGG_00065 [Chaetomium globosum CBS 148.51]	3528	XP_001219286	65
Contig_28_consensus_sequence:g223.t1	serine proteinase	894	XP_001219287	55

Contig_28_consensus_sequence:g224.t1	hypothetical protein CHGG_00067 [Chaetomium globosum CBS 148.51]	4134	XP_001219288	70
Contig_28_consensus_sequence:g225.t1	transcriptional activator srcap-like protein	5028	XP_001219289	62
Contig_31_consensus_sequence:g226.t1	nmra-like family protein	789	EGU88410	66
Contig_31_consensus_sequence:g227.t1	short-chain dehydrogenase reductase family	777	AEO64460	79
Contig_31_consensus_sequence:g228.t1	reverse transcriptase	738	EGU86710	65
Contig_31_consensus_sequence:g229.t1	cytochrome p450	1548	XP_003039677	70
Contig_31_consensus_sequence:g229.t2	cytochrome p450	1497	XP_003039677	68
Contig_31_consensus_sequence:g230.t1	short chain	804	XP_003039289	68
Contig_31_consensus_sequence:g231.t1	caib baif family enzyme	1764	XP_003039162	75
Contig_31_consensus_sequence:g232.t1	dienelactone hydrolase family protein	783	XP_002483450	48
Contig_31_consensus_sequence:g232.t2	dienelactone hydrolase family protein	771	XP_002483450	48
Contig_31_consensus_sequence:g233.t1	flavin-containing monooxygenase	1728	XP_003039158	75
Contig_31_consensus_sequence:g234.t1	x-pro dipeptidyl-peptidase c-terminal non-catalytic domain-containing protein	1806	XP_003043289	62
Contig_31_consensus_sequence:g234.t2	x-pro dipeptidyl-peptidase c-terminal non-catalytic domain-containing protein	1779	XP_003043289	63
Contig_31_consensus_sequence:g235.t1	short chain dehydrogenase family	729	XP_002147372	64
Contig_31_consensus_sequence:g236.t1	benzoate 4-monooxygenase cytochrome	621	AEO68593	53
Contig_31_consensus_sequence:g236.t2	benzoate 4-monooxygenase cytochrome	666	AEO68593	57
Contig_30_consensus_sequence:g237.t1	heterokaryon incompatibility protein	2073	XP_001823679	44
Contig_30_consensus_sequence:g238.t1	hypothetical protein NECHADRAFT_87170 [Nectria haematococca mpVI 77-13-4]	699	XP_003041750	48
Contig_30_consensus_sequence:g239.t1	predicted protein [Verticillium albo-atrum VaMs.102]	684	XP_003000052	47
Contig_30_consensus_sequence:g240.t1	methyltransferase domain-containing protein	741	XP_003044732	77
Contig_30_consensus_sequence:g241.t1	protein	1551	XP_002545148	57
Contig_30_consensus_sequence:g242.t1	f-box domain protein	1590	EGU87702	88
Contig_30_consensus_sequence:g243.t1	hypothetical protein FOXB_07703 [Fusarium oxysporum Fo5176]	441	EGU81779	100
Contig_32_consensus_sequence:g244.t1	c6 finger domain	1137	EGU74233	94
Contig_32_consensus_sequence:g245.t1	dienelactone hydrolase family protein	846	EGU74234	92
Contig_32_consensus_sequence:g246.t1	c6 transcription	1782	EGU74235	88
Contig_32_consensus_sequence:g247.t1	alpha- -glucanase	3399	EGU74236	99
Contig_32_consensus_sequence:g248.t1	von willebrand factor	828	EGU74237	100
Contig_32_consensus_sequence:g249.t1	metabolite transport	1518	EGU74238	99
Contig_32_consensus_sequence:g250.t1	extracellular gdsl-like lipase	1221	EGU74239	97
Contig_32_consensus_sequence:g251.t1	alpha-glucuronidase	2505	EGU79249	99
Contig_32_consensus_sequence:g252.t1	mfs maltose permease	1431	EGU79250	99
Contig_39_consensus_sequence:g253.t1	hypothetical protein FOXB_02334 [Fusarium oxysporum Fo5176]	720	EGU87156	84
Contig_39_consensus_sequence:g253.t2	hypothetical protein FOXB_02334 [Fusarium oxysporum Fo5176]	729	EGU87156	82
Contig_39_consensus_sequence:g254.t1	nad dependent epimerase	921	XP_003054026	70
Contig_39_consensus_sequence:g255.t1	monooxygenase	1626	CAP58783	52
Contig_39_consensus_sequence:g255.t2	flavin-binding monooxygenase	1590	CAP58783	51

Contig_39_consensus_sequence:g256.t1	para-nitrobenzyl esterase	1578	XP_003054728	66
Contig_39_consensus_sequence:g257.t1	beta-lactamase	1053	BAE65046	57
Contig_39_consensus_sequence:g258.t1	beta-lactamase	1182	EGU73355	94
Contig_34_consensus_sequence:g259.t1	protein	978	EGU88713	99
Contig_34_consensus_sequence:g260.t1	terpene synthase metal binding domain protein	2484	EGU88712	98
Contig_34_consensus_sequence:g260.t2	terpene synthase metal binding domain protein	2196	EGU88712	98
Contig_34_consensus_sequence:g261.t1	transcriptional regulator	1386	EGU77249	100
Contig_34_consensus_sequence:g262.t1	cas1 appressorium specific protein	906	EGU77248	99
Contig_34_consensus_sequence:g263.t1	n. i.	810	EGU77247	100
Contig_34_consensus_sequence:g264.t1	mfs transporter	1440	EGU77246	98
Contig_34_consensus_sequence:g264.t2	mfs transporter	1500	XP_391695	97
Contig_34_consensus_sequence:g265.t1	early growth response protein 1 (egr-1)	1971	EGU77245	99
Contig_34_consensus_sequence:g266.t1	translation initiation	351	EGU77244	100
Contig_34_consensus_sequence:g267.t1	gibberellin 3-beta	1206	EGU77243	100
Contig_42_consensus_sequence:g268.t1	mads-box transcription factor pvg4	990	EGU76626	97
Contig_42_consensus_sequence:g269.t1	endo- -beta-	984	EGU76230	100
Contig_42_consensus_sequence:g270.t1	hypothetical protein FOXB_12491 [Fusarium oxysporum Fo5176]	621	EGU76999	98
Contig_43_consensus_sequence:g272.t1	protein	2217	XP_386797	45
Contig_43_consensus_sequence:g273.t1	hypothetical protein FOXB_14162 [Fusarium oxysporum Fo5176]	510	EGU75317	50
Contig_40_consensus_sequence:g274.t1	acetylornithine aminotransferase	833	EGU75102	99
Contig_40_consensus_sequence:g275.t1	hypothetical protein FOXB_14415 [Fusarium oxysporum Fo5176]	498	EGU75101	98
Contig_40_consensus_sequence:g276.t1	atp synthase f1	1968	EGU75100	94
Contig_40_consensus_sequence:g277.t1	lysophospholipase	1947	EGU75099	100
Contig_40_consensus_sequence:g278.t1	transcription factor cys6	1473	EGU75098	99
Contig_40_consensus_sequence:g278.t2	transcription factor cys6	1518	EGU75098	99
Contig_40_consensus_sequence:g279.t1	peptidase dimerization domain protein	1542	EGU75094	98
Contig_40_consensus_sequence:g280.t1	cell wall glycoprotein	897	EGU75093	99
Contig_40_consensus_sequence:g281.t1	aromatic-l-amino-acid decarboxylase	1479	EGU75092	100
Contig_41_consensus_sequence:g282.t1	3-alpha-(or 20-beta)-hydroxysteroid dehydrogenase	762	XP_747051	67
Contig_41_consensus_sequence:g283.t1	metallo-beta-lactamase superfamily protein	843	XP_001276413	83
Contig_41_consensus_sequence:g284.t1	mfs monocarboxylate transporter	564	EGR46192	82
Contig_41_consensus_sequence:g285.t1	fungal specific transcription factor	1446	XP_003044377	60
Contig_41_consensus_sequence:g285.t2	fungal specific transcription factor	1311	XP_003044377	60
Contig_41_consensus_sequence:g286.t1	abc multidrug	711	EGY14129	80
Contig_41_consensus_sequence:g287.t1	abc multidrug	588	XP_003044376	68
Contig_41_consensus_sequence:g288.t1	abc multidrug	279	XP_003044376	92
Contig_45_consensus_sequence:g289.t1	alcohol dehydrogenase	996	XP_003043160	76
Contig_45_consensus_sequence:g290.t1	nadh:flavin oxidoreductase nadh oxidase	1215	XP_003043159	95

Contig_45_consensus_sequence:g291.t1	metallo-beta-lactamase superfamily protein	1194	XP_003043075	86
Contig_45_consensus_sequence:g291.t2	predicted protein [Nectria haematococca mpVI 77-13-4]	648	XP_003043074	76
Contig_45_consensus_sequence:g291.t3	metallo-beta-lactamase superfamily protein	1842	XP_003043075	86
Contig_45_consensus_sequence:g292.t1	c2h2 transcription factor	2262	XP_003043076	87
Contig_45_consensus_sequence:g293.t1	hypothetical protein NECHADRAFT_86292 [Nectria haematococca mpVI 77-13-4]	975	XP_003043077	91
Contig_45_consensus_sequence:g294.t1	alcohol dehydrogenase	297	XP_003040675	70
Contig_45_consensus_sequence:g295.t1	alcohol dehydrogenase	1119	XP_003043161	89
Contig_45_consensus_sequence:g296.t1	mfs sugar transporter	1557	XP_003043162	93
Contig_37_consensus_sequence:g297.t1	fot5 transposase	525	EGU83272	100
Contig_37_consensus_sequence:g298.t1	amidohydrolase	1134	XP_003039583	92
Contig_37_consensus_sequence:g299.t1	cp2 transcription factor	1020	EGU88551	98
Contig_37_consensus_sequence:g299.t2	cp2 transcription	1065	EGU88551	90
Contig_37_consensus_sequence:g300.t1	c6 transcription	1605	EGU88550	99
Contig_37_consensus_sequence:g300.t2	c6 transcription	1725	EGU88550	99
Contig_44_consensus_sequence:g301.t1	hypothetical protein FOXB_14159 [Fusarium oxysporum Fo5176]	1152	EGU75314	61
Contig_44_consensus_sequence:g302.t1	kinesin k39	2412	XP_816749	41
Contig_44_consensus_sequence:g303.t1	repeat organellar	1914	XP_003046878	44
Contig_44_consensus_sequence:g304.t1	protein	1253	XP_003046880	58
Contig_54_consensus_sequence:g306.t1	hypothetical protein FOXB_16635 [Fusarium oxysporum Fo5176]	1818	EGU72855	98
Contig_54_consensus_sequence:g307.t1	hypothetical protein FOXB_11451 [Fusarium oxysporum Fo5176]	939	EGU78041	67
Contig_54_consensus_sequence:g308.t1	hypothetical protein FOXB_17311 [Fusarium oxysporum Fo5176]	1392	EGU72180	97
Contig_54_consensus_sequence:g309.t1	hypothetical protein FOXB_04928 [Fusarium oxysporum Fo5176]	1737	EGU84557	100
Contig_58_consensus_sequence:g310.t1	zinc-binding dehydrogenase	990	EGP92564	64
Contig_58_consensus_sequence:g310.t2	zinc-binding dehydrogenase	972	EGP92564	63
Contig_58_consensus_sequence:g311.t1	cytochrome p450	1653	AEO63946	61
Contig_58_consensus_sequence:g313.t1	amino acid	1578	EGX88424	51
Contig_58_consensus_sequence:g313.t2	amino acid	1569	EGX88424	52
Contig_58_consensus_sequence:g314.t1	cupin domain-containing protein	1044	XP_390814	57
Contig_58_consensus_sequence:g315.t1	carboxylesterase type b	1341	XP_001243164	63
Contig_58_consensus_sequence:g316.t1	n. i.	918	XP_003054727	59
Contig_50_consensus_sequence:g317.t1	binuclear zinc transcription factor	1752	XP_003039651	68
Contig_50_consensus_sequence:g317.t2	binuclear zinc transcription factor	1722	XP_003039651	68
Contig_50_consensus_sequence:g318.t1	mfs transporter	1647	XP_003039659	97
Contig_50_consensus_sequence:g318.t2	mfs transporter	1659	XP_003039659	96
Contig_50_consensus_sequence:g319.t1	aldehyde reductase ii	1047	XP_003039649	91
Contig_50_consensus_sequence:g320.t1	n. i.	927	XP_003039650	95
Contig_50_consensus_sequence:g321.t1	n. i.	936	XP_003039652	83
Contig_50_consensus_sequence:g322.t1	major facilitator superfamily transporter	1110	EGY18563	73

Contig_50_consensus_sequence:g322.t2	major facilitator superfamily transporter	1104	EGY18563	73
Contig_53_consensus_sequence:g323.t1	umta methyltransferase family protein	918	EGU71752	98
Contig_53_consensus_sequence:g324.t1	homoserine dehydrogenase	864	XP_002383445	70
Contig_53_consensus_sequence:g325.t1	succinate-semialdehyde dehydrogenase	492	XP_003045566	81
Contig_53_consensus_sequence:g326.t1	amino acid permease	1728	EFX02665	77
Contig_59_consensus_sequence:g327.t1	het domain protein	2244	XP_659244	43
Contig_59_consensus_sequence:g328.t1	het domain protein	2664	XP_002487088	46
Contig_59_consensus_sequence:g329.t1	hypothetical protein FOXB_01363 [Fusarium oxysporum Fo5176]	1479	EGU88115	51
Contig_59_consensus_sequence:g330.t2	predicted protein [Nectria haematococca mpVI 77-13-4]	450	XP_003040346	44
Contig_59_consensus_sequence:g331.t1	hypothetical protein VDAG_02263 [Verticillium dahliae VdLs.17]	1560	EGY20247	42
Contig_47_consensus_sequence:g332.t1	hypothetical protein VDAG_05170 [Verticillium dahliae VdLs.17]	1230	EGY23732	61
Contig_47_consensus_sequence:g333.t1	hypothetical protein FOXB_03765 [Fusarium oxysporum Fo5176]	972	EGU85718	100
Contig_47_consensus_sequence:g334.t1	hypothetical protein FOXB_03763 [Fusarium oxysporum Fo5176]	684	EGU85719	97
Contig_63_consensus_sequence:g335.t1	2-dehydropantoate 2-reductase family	975	EGU73027	95
Contig_63_consensus_sequence:g336.t1	nad dependent epimerase dehydratase family protein	504	EGU72449	95
Contig_63_consensus_sequence:g337.t1	2-dehydropantoate 2-	306	EGU83989	75
Contig_63_consensus_sequence:g338.t1	toxin biosynthesis	1218	XP_003047240	86
Contig_63_consensus_sequence:g338.t2	toxin biosynthesis	1311	XP_003047240	86
Contig_63_consensus_sequence:g339.t1	hypothetical protein FOXB_17042 [Fusarium oxysporum Fo5176]	1776	EGU72450	92
Contig_63_consensus_sequence:g340.t1	branched-chain-amino-acid aminotransferase	1185	XP_003039790	85
Contig_60_consensus_sequence:g341.t1	glutathione s-transferase-like protein	669	EGU74255	85
Contig_60_consensus_sequence:g342.t1	glucooligosaccharide	1494	XP_003045548	67
Contig_60_consensus_sequence:g343.t1	amidohydrolase family protein	1509	XP_003041457	70
Contig_60_consensus_sequence:g343.t2	amidohydrolase family protein	1488	XP_003041457	69
Contig_60_consensus_sequence:g344.t1	family protein	705	XP_001404526	70
Contig_60_consensus_sequence:g345.t1	tetratricopeptide repeat domain protein	1929	EGU82069	93
Contig_56_consensus_sequence:g346.t1	ww domain-containing oxidoreductase	1008	XP_003041650	83
Contig_56_consensus_sequence:g347.t1	quinone oxidoreductase	1053	XP_003041711	79
Contig_56_consensus_sequence:g347.t2	quinone oxidoreductase	1047	XP_003041711	80
Contig_56_consensus_sequence:g348.t1	protein	1770	XP_003041709	74
Contig_56_consensus_sequence:g349.t1	nadh:ubiquinone oxidoreductase subunit	1824	EGU77691	89
Contig_56_consensus_sequence:g349.t2	nadh:ubiquinone oxidoreductase subunit	1827	EGU77691	89
Contig_56_consensus_sequence:g350.t1	esterase lipase	909	XP_003047694	86
Contig_61_consensus_sequence:g351.t1	peptidase s8 and s53	2334	EGU86858	99
Contig_61_consensus_sequence:g352.t1	hypothetical protein FOXB_02621 [Fusarium oxysporum Fo5176]	1305	EGU86859	97
Contig_61_consensus_sequence:g352.t2	hypothetical protein FOXB_02621 [Fusarium oxysporum Fo5176]	1191	EGU86859	97
Contig_61_consensus_sequence:g353.t1	hypothetical protein FOXB_02622 [Fusarium oxysporum Fo5176]	1134	EGU86860	92
Contig_61_consensus_sequence:g354.t1	yr626_mimiv ame: full=uncharacterized protein r626	3000	EGU86861	98

Contig_61_consensus_sequence:g355.t1	ankyrin repeat protein	2442	XP_391601	71
Contig_61_consensus_sequence:g355.t2	ankyrin repeat protein	2559	XP_391601	71
Contig_71_consensus_sequence:g357.t1	zinc-binding dehydrogenase family	1158	XP_001228886	76
Contig_52_consensus_sequence:g358.t1	tannase and feruloyl esterase	1410	EGU81309	79
Contig_52_consensus_sequence:g358.t2	tannase and feruloyl esterase	1374	XP_003049335	82
Contig_52_consensus_sequence:g359.t1	feruloyl esterase b precursor	786	XP_003049335	56
Contig_52_consensus_sequence:g360.t1	lactose permease	1374	EGU75611	62
Contig_52_consensus_sequence:g361.t1	family taurine catabolism dioxygenase	3126	XP_003052909	71
Contig_52_consensus_sequence:g361.t2	family taurine catabolism dioxygenase	3138	XP_003052909	71
Contig_65_consensus_sequence:g362.t1	mfs sugar	1518	EGU86533	88
Contig_65_consensus_sequence:g362.t2	sugar transporter family protein	1452	EGU86533	92
Contig_65_consensus_sequence:g363.t1	hypothetical protein FOXB_02968 [Fusarium oxysporum Fo5176]	1026	EGU86534	99
Contig_65_consensus_sequence:g364.t1	2-deoxy-d-gluconate 3-dehydrogenase	591	EGU86535	98
Contig_65_consensus_sequence:g365.t1	cupin 2 domain-containing protein	540	EGU86536	98
Contig_65_consensus_sequence:g366.t1	n. i.	1689	XP_001558928	82
Contig_65_consensus_sequence:g367.t1	nadph dehydrogenase	1353	EGU84716	99
Contig_67_consensus_sequence:g368.t1	nmra-like family protein	1455	EGU86314	97
Contig_67_consensus_sequence:g369.t1	arylsulfatase	1767	EGU86314	99
Contig_67_consensus_sequence:g369.t2	arylsulfatase-like protein	1740	EGU86314	98
Contig_67_consensus_sequence:g370.t1	protein	1575	EGU86312	99
Contig_55_consensus_sequence:g371.t1	zinc finger	3762	XP_390745	72
Contig_55_consensus_sequence:g372.t1	snf2 family n-terminal domain containing protein	1665	XP_390744	75
Contig_66_consensus_sequence:g373.t1	polysaccharide biosynthesis protein vipa tvib	1350	XP_003045612	85
Contig_66_consensus_sequence:g374.t1	polysaccharide deacetylase	1326	XP_003045478	81
Contig_66_consensus_sequence:g375.t1	chitin synthase	1815	XP_003045479	80
Contig_64_consensus_sequence:g376.t1	nacht and ankyrin domain protein	3870	EGU80023	72
Contig_62_consensus_sequence:g377.t1	predicted protein [Nectria haematococca mpVI 77-13-4]	7755	XP_003040278	45
Contig_62_consensus_sequence:g378.t1	predicted protein [Laccaria bicolor S238N-H82]	480	XP_001880833	51
Contig_62_consensus_sequence:g379.t1	predicted protein [Trichoderma reesei QM6a]	954	EGR52441	40
Contig_62_consensus_sequence:g379.t2	predicted protein [Trichoderma reesei QM6a]	1086	EGR52441	40
Contig_70_consensus_sequence:g380.t1	c6 transcription	903	EGU86864	53
Contig_70_consensus_sequence:g381.t1	rta1 domain	756	XP_003040764	85
Contig_70_consensus_sequence:g382.t1	rta1 like protein	849	XP_391424	85
Contig_82_consensus_sequence:g383.t1	fad binding domain protein	1749	XP_003039076	75
Contig_82_consensus_sequence:g383.t2	fad binding domain protein	1752	XP_003039076	75
Contig_82_consensus_sequence:g384.t1	short chain	801	XP_003039670	83
Contig_82_consensus_sequence:g385.t1	cytochrome p450	1611	XP_003039677	82
Contig_82_consensus_sequence:g386.t1	c2h2 type zinc finger domain protein	2058	XP_003039669	52

Contig_82_consensus_sequence:g386.t2	c2h2 type zinc finger domain protein	2316	XP_003039669	55
Contig_73_consensus_sequence:g387.t1	cytochrome p450	1395	BAK09386	55
Contig_73_consensus_sequence:g387.t2	cytochrome p450	1295	BAK09386	55
Contig_73_consensus_sequence:g388.t1	short-chain dehydrogenase reductase sdr	855	XP_001218628	78
Contig_73_consensus_sequence:g389.t1	hypothetical e protein	459	XP_003044498	61
Contig_73_consensus_sequence:g390.t1	beta-lactamase	1149	XP_003044375	77
Contig_73_consensus_sequence:g391.t1	bzip transcription	1974	XP_001218629	58
Contig_73_consensus_sequence:g391.t2	bzip transcription	1995	XP_001218629	58
Contig_73_consensus_sequence:g392.t1	alpha beta hydrolase fold-3 domain protein	1041	XP_001218624	64
Contig_73_consensus_sequence:g393.t1	flavin-binding	1932	EGP87984	62
Contig_89_consensus_sequence:g394.t1	prib protein	1971	EGU84437	98
Contig_89_consensus_sequence:g394.t2	prib protein	1965	EGU84437	98
Contig_89_consensus_sequence:g395.t1	hypothetical protein FOXB_12734 [Fusarium oxysporum Fo5176]	1128	EGU76758	98
Contig_89_consensus_sequence:g396.t1	glycosyltransferase family 28 domain-containing protein	2226	EGU76757	98
Contig_89_consensus_sequence:g397.t1	hypothetical protein FOXB_12732 [Fusarium oxysporum Fo5176]	3057	EGU76756	99
Contig_57_consensus_sequence:g398.t1	arrestin domain-containing protein	700	XP_003040586	80
Contig_57_consensus_sequence:g399.t1	formylmethionine deformylase-like protein	1926	EGU85724	83
Contig_57_consensus_sequence:g399.t2	hypothetical protein FOXB_03757 [Fusarium oxysporum Fo5176]	1959	EGU85724	88
Contig_57_consensus_sequence:g400.t1	hypothetical protein FOXB_02699 [Fusarium oxysporum Fo5176]	1737	EGU86783	96
Contig_57_consensus_sequence:g401.t1	hypothetical protein FOXB_02698 [Fusarium oxysporum Fo5176]	1185	EGU86784	95
Contig_78_consensus_sequence:g402.t1	hypothetical protein FOXB_07880 [Fusarium oxysporum Fo5176]	802	EGU81607	78
Contig_78_consensus_sequence:g403.t1	hypothetical protein FG04519.1 [Gibberella zeae PH-1]	2565	XP_384695	59
Contig_78_consensus_sequence:g403.t2	hypothetical protein FG04519.1 [Gibberella zeae PH-1]	2508	XP_384695	60
Contig_78_consensus_sequence:g404.t1	monooxygenase	1470	EGU81932	86
Contig_78_consensus_sequence:g405.t1	fungal specific transcription	1578	EGU81930	78
Contig_80_consensus_sequence:g406.t1	Pc13g00190 [Penicillium chrysogenum Wisconsin 54-1255]	1334	XP_002558473	81
Contig_80_consensus_sequence:g406.t2	Pc13g00600 [Penicillium chrysogenum Wisconsin 54-1255]	1292	XP_002558509	75
Contig_80_consensus_sequence:g407.t1	hypothetical protein FOXB_05177 [Fusarium oxysporum Fo5176]	3891	EGU84309	65
Contig_80_consensus_sequence:g408.t1	protein	2238	EGU83019	94
Contig_80_consensus_sequence:g408.t2	protein	2193	EGU83019	92
Contig_74_consensus_sequence:g409.t1	hypothetical protein FOXB_03744 [Fusarium oxysporum Fo5176]	1809	EGU85740	97
Contig_74_consensus_sequence:g410.t1	serine threonine-protein phosphatase 6 regulatory ankyrin repeat subunit a-like	1218	EGU85739	97
Contig_75_consensus_sequence:g411.t1	centromere microtubule binding protein cbf5-like protein	1227	XP_003050413	66
Contig_75_consensus_sequence:g412.t1	carbon-nitrogen hydrolase	3078	XP_003050415	59
Contig_75_consensus_sequence:g412.t2	carbon-nitrogen hydrolase	3084	XP_003050415	59
Contig_75_consensus_sequence:g413.t1	exo-alpha-sialidase neuraminidase	1956	EGU77650	75
Contig_75_consensus_sequence:g413.t2	exo-alpha-sialidase neuraminidase	1683	EGU77650	75
Contig_83_consensus_sequence:g414.t1	hypothetical protein PSYAC_25013 [Pseudomonas syringae pv. actinidiae str. M302091]	393	EGH68103	81

Contig_83_consensus_sequence:g415.t1	alcohol dehydrogenase	1083	XP_003043158	80
Contig_83_consensus_sequence:g416.t1	beta-lactamase	1260	XP_003049512	76
Contig_83_consensus_sequence:g417.t1	amino acid	1590	XP_003049271	86
Contig_83_consensus_sequence:g417.t2	amino acid	1581	XP_003049271	87
Contig_84_consensus_sequence:g418.t1	het domain protein	2646	XP_002840035	41
Contig_84_consensus_sequence:g419.t1	hypothetical protein FG11035.1 [Gibberella zeae PH-1]	522	XP_391211	64
Contig_90_consensus_sequence:g420.t1	hypothetical protein FOXB_16501 [Fusarium oxysporum Fo5176]	501	EGU72991	52
Contig_90_consensus_sequence:g421.t1	hypothetical protein FOXB_15642 [Fusarium oxysporum Fo5176]	2205	EGU73847	53
Contig_90_consensus_sequence:g421.t2	hypothetical protein FOXB_15642 [Fusarium oxysporum Fo5176]	2178	EGU73847	53
Contig_90_consensus_sequence:g422.t1	hypothetical protein NECHADRAFT_82228 [Nectria haematococca mpVI 77-13-4]	2226	XP_003041506	56
Contig_90_consensus_sequence:g423.t1	hypothetical protein FOXB_17318 [Fusarium oxysporum Fo5176]	1854	EGU72173	73
Contig_104_consensus_sequence:g424.t1	alcohol dehydrogenase	1041	XP_003049085	88
Contig_104_consensus_sequence:g424.t2	alcohol dehydrogenase	1065	XP_003049085	90
Contig_104_consensus_sequence:g425.t1	c-5 cytosine methyltransferase	1287	XP_003049333	59
Contig_104_consensus_sequence:g426.t1	hypothetical protein BC1G_16291 [Botryotinia fuckeliana B05.10]	690	XP_001545194	70
Contig_104_consensus_sequence:g427.t1	rta1 domain	882	XP_001545295	85
Contig_95_consensus_sequence:g428.t1	c6 transcription	1976	EGR46936	53
Contig_95_consensus_sequence:g428.t2	c6 transcription	2003	EGR46936	55
Contig_95_consensus_sequence:g429.t1	monocarboxylate	2220	XP_390682	78
Contig_95_consensus_sequence:g430.t1	c6 zinc finger domain-containing protein	1422	XP_003043448	64
Contig_105_consensus_sequence:g431.t1	cysteine proteinase	1569	EGU86849	99
Contig_105_consensus_sequence:g432.t1	hypothetical protein FOXB_02321 [Fusarium oxysporum Fo5176]	630	EGU87161	98
Contig_105_consensus_sequence:g433.t1	protein kinase-like domain	1002	EGU87162	96
Contig_105_consensus_sequence:g434.t1	hypothetical protein FOXB_06697 [Fusarium oxysporum Fo5176]	684	EGU82790	98
Contig_103_consensus_sequence:g435.t1	hypothetical protein FOXB_02640 [Fusarium oxysporum Fo5176]	650	EGU86837	98
Contig_103_consensus_sequence:g435.t2	hypothetical protein FOXB_02640 [Fusarium oxysporum Fo5176]	567	EGU86837	98
Contig_103_consensus_sequence:g436.t1	hypothetical protein FOXB_02650 [Fusarium oxysporum Fo5176]	906	EGU86832	82
Contig_103_consensus_sequence:g437.t1	hypothetical protein FOXB_02674 [Fusarium oxysporum Fo5176]	852	EGU86806	59
Contig_103_consensus_sequence:g438.t1	hypothetical protein FOXB_15416 [Fusarium oxysporum Fo5176]	1293	EGU74071	99
Contig_93_consensus_sequence:g439.t1	vitamin h	453	XP_388192	95
Contig_93_consensus_sequence:g439.t2	vitamin h	369	XP_388192	95
Contig_93_consensus_sequence:g440.t1	beta-galactosidase	2691	XP_663992	74
Contig_93_consensus_sequence:g441.t1	ankyrin repeat-containing	6089	EGU80426	88
Contig_92_consensus_sequence:g442.t1	pectin lyase	1134	EGU86866	100
Contig_92_consensus_sequence:g443.t1	family 12 glycoside hydrolase	1116	EGU75806	98
Contig_92_consensus_sequence:g444.t1	hypothetical protein FOXB_13717 [Fusarium oxysporum Fo5176]	942	EGU75805	94
Contig_92_consensus_sequence:g445.t1	related to small s protein	2883	EGU75804	96
Contig_92_consensus_sequence:g446.t1	rta1 domain	609	EGU75803	98

Contig_102_consensus_sequence:g447.t1	mfs transporter	1059	XP_001799387	73
Contig_102_consensus_sequence:g447.t2	mfs transporter	1113	EGU72359	95
Contig_102_consensus_sequence:g448.t1	protein	744	EGU83902	94
Contig_102_consensus_sequence:g449.t1	sam-dependent methyltransferase coq5 family protein	783	EGU83903	95
Contig_102_consensus_sequence:g449.t2	sam-dependent methyltransferase coq5 family protein	840	EGU83903	95
Contig_102_consensus_sequence:g450.t1	hypothetical protein FOXB_15531 [Fusarium oxysporum Fo5176]	1227	EGU73968	87
Contig_98_consensus_sequence:g451.t1	alpha beta hydrolase domain-containing protein	732	EGU74360	97
Contig_98_consensus_sequence:g451.t2	alpha beta hydrolase domain-containing protein	786	EGU74360	97
Contig_98_consensus_sequence:g452.t1	c2h2 type zinc finger containing protein	1110	EGU71718	98
Contig_98_consensus_sequence:g453.t1	galactoside o-acetyltransferase	549	EGU71720	79
Contig_98_consensus_sequence:g454.t1	pisatin demethylase	1491	AAR32716	90
Contig_119_consensus_sequence:g455.t1	hypothetical protein NECHADRAFT_80893 [Nectria haematococca mpVI 77-13-4]	1503	XP_003051446	60
Contig_119_consensus_sequence:g455.t2	hypothetical protein NECHADRAFT_80893 [Nectria haematococca mpVI 77-13-4]	1530	XP_003051446	60
Contig_119_consensus_sequence:g456.t1	hypothetical protein CHGG_01009 [Chaetomium globosum CBS 148.51]	849	XP_001220230	44
Contig_119_consensus_sequence:g457.t1	tetratricopeptide repeat protein	3471	EGU79305	54
Contig_68_consensus_sequence:g458.t1	ankyrin repeat domain-containing protein 52	3375	EGU78775	87
Contig_68_consensus_sequence:g458.t2	ankyrin repeat domain-containing protein 52	3327	EGU78775	85
Contig_68_consensus_sequence:g459.t1	protein kinase-like domain	2439	EGY23756	69
Contig_118_consensus_sequence:g460.t1	methyltransferase domain-containing protein	667	EGU73857	83
Contig_118_consensus_sequence:g460.t2	methyltransferase domain-containing protein	625	EGU73857	82
Contig_118_consensus_sequence:g461.t1	hypothetical protein FOXB_15637 [Fusarium oxysporum Fo5176]	900	EGU73856	98
Contig_118_consensus_sequence:g462.t1	hypothetical protein VDAG_05225 [Verticillium dahliae VdLs.17]	312	EGY23787	80
Contig_118_consensus_sequence:g463.t1	hypothetical protein VDAG_09127 [Verticillium dahliae VdLs.17]	1296	EGY18601	61
Contig_118_consensus_sequence:g464.t1	alpha-galactosidase a	834	EGU73855	99
Contig_118_consensus_sequence:g465.t1	arsenic resistance protein	1509	EGU83904	95
Contig_115_consensus_sequence:g466.t1	hypothetical protein NECHADRAFT_16076 [Nectria haematococca mpVI 77-13-4]	858	XP_003046174	76
Contig_115_consensus_sequence:g466.t2	hypothetical protein NECHADRAFT_16076 [Nectria haematococca mpVI 77-13-4]	1029	XP_003046174	78
Contig_115_consensus_sequence:g467.t1	alpha beta hydrolase fold-3 domain protein	891	XP_002378531	54
Contig_115_consensus_sequence:g468.t1	lignostilbene dioxygenase family protein	810	XP_001823663	58
Contig_115_consensus_sequence:g468.t2	lignostilbene dioxygenase family protein	1020	XP_001823663	52
Contig_115_consensus_sequence:g469.t1	c6 transcription factor	1512	XP_002560094	58
Contig_106_consensus_sequence:g470.t1	hypothetical protein FOXB_04997 [Fusarium oxysporum Fo5176]	2661	EGU84489	52
Contig_106_consensus_sequence:g470.t2	hypothetical protein FOXB_04997 [Fusarium oxysporum Fo5176]	2622	EGU84489	50
Contig_106_consensus_sequence:g471.t1	ankyrin repeat protein	2364	XP_003174550	47
Contig_106_consensus_sequence:g473.t1	hypothetical protein FOXB_02610 [Fusarium oxysporum Fo5176]	354	EGU86900	99
Contig_116_consensus_sequence:g474.t1	tpa: (afu_orthologue afua_7g06960)	639	XP_003071474	49
Contig_116_consensus_sequence:g475.t1	cutinase transcription factor 1 beta	1935	EFQ29770	64
Contig_116_consensus_sequence:g476.t1	mfs transporter	1311	XP_384950	64

Contig_116_consensus_sequence:g476.t2	mfs transporter	1260	XP_384950	63
Contig_111_consensus_sequence:g477.t1	calpain-1 catalytic subunit	2847	EGU86849	86
Contig_111_consensus_sequence:g478.t1	hypothetical protein FOXB_15642 [Fusarium oxysporum Fo5176]	1752	EGU73847	39
Contig_111_consensus_sequence:g479.t1	hypothetical protein FOXB_17511 [Fusarium oxysporum Fo5176]	633	EGU71980	51
Contig_94_consensus_sequence:g480.t1	domain-containing protein	1881	XP_003042569	68
Contig_94_consensus_sequence:g480.t2	domain-containing protein	1857	XP_003042569	68
Contig_94_consensus_sequence:g481.t1	bacteriodes thetaiotaomicron symbiotic	4509	XP_003042656	76
Contig_94_consensus_sequence:g481.t2	bacteriodes thetaiotaomicron symbiotic	4527	XP_003042656	76
Contig_112_consensus_sequence:g482.t1	abc transporter	4881	EGU72509	95
Contig_112_consensus_sequence:g482.t2	abc transporter	4680	EGU72509	99
Contig_123_consensus_sequence:g483.t1	hypothetical protein FOXB_02711 [Fusarium oxysporum Fo5176]	420	EGU86771	100
Contig_123_consensus_sequence:g484.t1	protein	2157	EGU86772	99
Contig_123_consensus_sequence:g485.t1	protein	555	EGU73040	100
Contig_117_consensus_sequence:g487.t1	cutinase transcription factor 1 beta	492	EGX89532	52
Contig_117_consensus_sequence:g488.t1	x-pro dipeptidyl-peptidase (s15 family) protein	3687	BAE59727	84
Contig_117_consensus_sequence:g488.t2	x-pro dipeptidyl-peptidase (s15 family) protein	3924	BAE59727	84
Contig_120_consensus_sequence:g489.t1	hypothetical protein FOXB_15475 [Fusarium oxysporum Fo5176]	170	EGU74024	69
Contig_120_consensus_sequence:g490.t1	hypothetical protein SELMODRAFT_427523 [Selaginella moellendorffii]	801	XP_002988886	54
Contig_120_consensus_sequence:g491.t1	cfem domain	906	XP_003042439	60
Contig_120_consensus_sequence:g491.t2	cfem domain-containing protein	900	XP_003042439	61
Contig_120_consensus_sequence:g492.t1	het-domain-containing protein	2433	EGO57402	45
Contig_120_consensus_sequence:g493.t1	mosquitocidal toxin	318	EFY84486	69
Contig_114_consensus_sequence:g494.t1	c6 transcription	771	EFY96470	59
Contig_114_consensus_sequence:g495.t1	class ii aldolase adducin domain protein	831	EGU87391	84
Contig_114_consensus_sequence:g496.t1	proline-specific permease	1383	EGU87393	76
Contig_114_consensus_sequence:g496.t2	proline-specific permease	1479	EGU87393	73
Contig_114_consensus_sequence:g497.t1	cystathionine beta-lyase	1209	EGU87394	79
Contig_114_consensus_sequence:g498.t1	transcriptional	738	EGU80329	84
Contig_137_consensus_sequence:g499.t1	prolyl-trna synthetase	3217	XP_003047584	73
Contig_137_consensus_sequence:g499.t2	prolyl-trna synthetase	3334	XP_003047584	71
Contig_137_consensus_sequence:g500.t1	mitochondrial carrier protein	903	XP_003047587	80
Contig_137_consensus_sequence:g500.t2	mitochondrial carrier protein	891	XP_003047587	80
Contig_137_consensus_sequence:g501.t1	delta-1-pyrroline-5-carboxylate dehydrogenase	1713	AEO65880	76
Contig_122_consensus_sequence:g502.t1	choline dehydrogenase	365	EGU74706	98
Contig_122_consensus_sequence:g503.t1	predicted protein [Nectria haematococca mpVI 77-13-4]	486	XP_003041185	72
Contig_122_consensus_sequence:g504.t1	predicted protein [Nectria haematococca mpVI 77-13-4]	1365	XP_003047053	41
Contig_122_consensus_sequence:g506.t1	predicted protein [Nectria haematococca mpVI 77-13-4]	794	XP_003041190	61
Contig_122_consensus_sequence:g506.t2	predicted protein [Nectria haematococca mpVI 77-13-4]	773	XP_003041190	61

APPENDIX VIII

Table 41 Presence of published motifs 41 candidate effector proteins of *Fusarium oxysporum* f. sp. *cepae* isolate Fus2.

Sequence Name	Total Times Found	Positions	Found As
>Contig_6_consensus_sequence_g66.t1 ; MatureChain: 26-1075	1	[399]	['RVLR']
>Contig_6_consensus_sequence_g66.t2 ; MatureChain: 26-1066	1	[399]	['RVLR']
>Contig_60_consensus_sequence_g343.t1 ; MatureChain: 18-502	1	[359]	['RALR']
>Contig_60_consensus_sequence_g343.t2 ; MatureChain: 18-495	1	[352]	['RALR']
>Contig_75_consensus_sequence_g413.t2 ; MatureChain: 23-560	1	[479]	['RELR']
>Contig_118_consensus_sequence_g463.t1 ; MatureChain: 19-431	1	[18]	['RQLR']
>Contig_106_consensus_sequence_g470.t1 ; MatureChain: 22-886	1	[724]	['RVLR']
>Contig_106_consensus_sequence_g470.t2 ; MatureChain: 22-873	1	[711]	['RVLR']

Sequence Name	Total Times Found	Positions	Found As
>Contig_6_consensus_sequence_g66.t1 ; MatureChain: 26-1075	1	[679]	['EER']
>Contig_6_consensus_sequence_g66.t2 ; MatureChain: 26-1066	1	[679]	['EER']
>Contig_23_consensus_sequence_g191.t1 ; MatureChain: 19-1836	1	[1779]	['EER']
>Contig_23_consensus_sequence_g192.t1 ; MatureChain: 28-623	1	[564]	['EER']
>Contig_39_consensus_sequence_g256.t1 ; MatureChain: 23-525	1	[168]	['EER']
>Contig_83_consensus_sequence_g416.t1 ; MatureChain: 24-419	1	[392]	['EER']
>Contig_106_consensus_sequence_g470.t1 ; MatureChain: 22-886	1	[255]	['EER']
>Contig_106_consensus_sequence_g470.t2 ; MatureChain: 22-873	1	[255]	['EER']

Sequence Name	Total Times Found	Positions	Found As
>Contig_2_consensus_sequence_g14.t2 ; MatureChain: 21-1153	1	[132]	['YGC']
>Contig_7_consensus_sequence_g71.t1 ; MatureChain: 19-336	1	[83]	['WGC']
>Contig_23_consensus_sequence_g191.t1 ; MatureChain: 19-1836	1	[1369]	['FGC']
>Contig_23_consensus_sequence_g192.t1 ; MatureChain: 28-623	1	[568]	['FCC']
>Contig_24_consensus_sequence_g197.t1 ; MatureChain: 25-531	1	[238]	['WGC']
>Contig_32_consensus_sequence_g247.t1 ; MatureChain: 19-1132	7	[452 499 552 599 637 720 734]	['FTC' 'YAC' 'FSC' 'FAC' 'WNC' 'YDC' 'FKC']
>Contig_34_consensus_sequence_g262.t1 ; MatureChain: 21-301	1	[98]	['YTC']
>Contig_40_consensus_sequence_g277.t1 ; MatureChain: 17-648	1	[477]	['FGC']
>Contig_47_consensus_sequence_g333.t1 ; MatureChain: 24-323	1	[204]	['FGC']
>Contig_66_consensus_sequence_g374.t1 ; MatureChain: 19-441	1	[349]	['YTC']
>Contig_57_consensus_sequence_g401.t1 ; MatureChain: 22-394	1	[143]	['YRC']
>Contig_106_consensus_sequence_g470.t1 ; MatureChain: 22-886	1	[10]	['YNC']
>Contig_106_consensus_sequence_g470.t2 ; MatureChain: 22-873	1	[10]	['YNC']
>Contig_94_consensus_sequence_g480.t1 ; MatureChain: 19-626	5	[302 337 436 523 601]	['WSC' 'YYC' 'YVC' 'YAC' 'WAC']
>Contig_94_consensus_sequence_g480.t2 ; MatureChain: 19-618	5	[294 329 428 515 593]	['WSC' 'YYC' 'YVC' 'YAC' 'WAC']
>Contig_94_consensus_sequence_g481.t1 ; MatureChain: 34-1502	2	[37 1067]	['YTC' 'FSC']
>Contig_94_consensus_sequence_g481.t2 ; MatureChain: 34-1508	2	[37 1073]	['YTC' 'FSC']

Sequence Name	Total Times Found	Positions	Found As
>Contig_24_consensus_sequence_g199.t1 ; MatureChain: 21-413	1	[39]	['HRRSL']
>Contig_27_consensus_sequence_g203.t1 ; MatureChain: 22-480	1	[126]	['HQRNL']
>Contig_83_consensus_sequence_g416.t1 ; MatureChain: 24-419	1	[155]	['HIRNL']

Sequence Name	Total Times Found	Positions	Found As
>Contig_23_consensus_sequence_g191.t1 ; MatureChain: 19-1836	1	[1758]	['GILR']
>Contig_27_consensus_sequence_g209.t1 ; MatureChain: 20-340	1	[205]	['GFLR']
>Contig_57_consensus_sequence_g400.t1 ; MatureChain: 19-578	1	[211]	['GFTR']

Sequence Name	Total Times Found	Positions	Found As
>Contig_23_consensus_sequence_g191.t1 ; MatureChain: 19-1836	1	[86]	['LQAR']
>Contig_67_consensus_sequence_g369.t1 ; MatureChain: 19-588	1	[362]	['IKAR']
>Contig_67_consensus_sequence_g369.t2 ; MatureChain: 19-579	1	[362]	['IKAR']
>Contig_106_consensus_sequence_g470.t1 ; MatureChain: 22-886	1	[846]	['LIAR']
>Contig_106_consensus_sequence_g470.t2 ; MatureChain: 22-873	1	[833]	['LIAR']

Sequence Name	Total Times Found	Positions	Found As
>Contig_75_consensus_sequence_g411.t1 ; MatureChain: 20-408	1	[145]	['DWL']
>Contig_92_consensus_sequence_g443.t1 ; MatureChain: 20-371	1	[308]	['DWL']
>Contig_106_consensus_sequence_g470.t1 ; MatureChain: 22-886	1	[279]	['DWL']
>Contig_106_consensus_sequence_g470.t2 ; MatureChain: 22-873	1	[279]	['DWL']

Sequence Name	Total Times Found	Positions	Found As
>Contig_2_consensus_sequence_g8.t1 ; MatureChain: 22-124	1	[91]	['RRIR']
>Contig_2_consensus_sequence_g14.t1 ; MatureChain: 21-1004	18	[24 113 164 211 263 271 312 347 465 563 570 580 629 672 848 896 902 976]	['RYWL' 'KPLT' 'RLIG' 'KDYK' 'KNLL' 'RQLL' 'KYLP' 'KYLQ' 'HGMT' 'KTFK' 'KILD' 'RALI' 'KNIS' 'KNLY' 'KTLQ' 'RAWV' 'KQLP' 'KCLR']
>Contig_2_consensus_sequence_g14.t2 ; MatureChain: 21-1153	24	[6 43 58 81 118 130 173 262 313 360 412 420 461 496 614 712 719 729 778 821 997 1045 1051 1125]	['REIP' 'KCYC' 'KHID' 'RHYY' 'KDMW' 'RLYG' 'RYWL' 'KPLT' 'RLIG' 'KDYK' 'KNLL' 'RQLL' 'KYLP' 'KYLQ' 'HGMT' 'KTFK' 'KILD' 'RALI' 'KNIS' 'KNLY' 'KTLQ' 'RAWV' 'KQLP' 'KCLR']
>Contig_5_consensus_sequence_g34.t1 ; MatureChain: 20-1063	18	[82 98 112 235 296 365 396 417 444 470 489 515 652 728 769 797 856 863]	['HHFS' 'KIIV' 'KLIK' 'HEFY' 'RTFY' 'REIL' 'RGLV' 'RRLP' 'RNYK' 'RRIW' 'HFMD' 'REFG' 'HMLD' 'RYFD' 'RVYI' 'RGFE' 'HTIK' 'RTLA']
>Contig_6_consensus_sequence_g66.t1 ; MatureChain: 26-1075	38	[65 73 90 110 121 138 169 241 247 289 303 309 315 348 359 399	['HSYC' 'KIYK' 'KSIR' 'KPMS' 'RTIS' 'RGYK' 'RFLP' 'RHYE' 'RHIG' 'HRWT'

		422 447 452	'HSLL' 'RIMI'
		494 504 525	'HSYP' 'RKIL'
		591 597 612	'HILL' 'RVLR'
		670 690 703	'KYLR' 'RVWE'
		751 761 768	'HPFS' 'KEMA'
		797 887 900	'RQLG' 'RGMT'
		914 952 995	'KPLG' 'KLYW'
		1037]	'KSMI' 'HAMC'
			'HYML' 'KDIA'
			'KKLG' 'KEYE'
			'KSFL' 'KDFT'
			'KRME' 'HIIQ'
			'HHFG' 'HAWK'
			'KYFQ' 'KSLQ']
			['HSYC' 'KIYK'
			'KSIR' 'KPMS'
			'RTIS' 'RGYK'
			'RFLP' 'RHYE'
			'RHIG' 'HRWT'
			'HSLL' 'RIMI'
		[65 73 90 110	'HSYP' 'RKIL'
		121 138 169	'HILL' 'RVLR'
		241 247 289	'KYLR' 'RVWE'
		303 309 315	'HPFS' 'KEMA'
		348 359 399	'RQLG' 'RGMT'
		422 447 452	'KPLG' 'KLYW'
		494 504 525	'KSMI' 'HAMC'
		591 597 612	'HYML' 'KDIA'
		670 690 703	'KKLG' 'KEYE'
		742 752 759	'KSFL' 'KDFT'
		788 878 891	'KRME' 'HIIQ'
		905 943 986	'HHFG' 'HAWK'
>Contig_6_consensus_sequence_g66.t2 ; MatureChain: 26-1066	38	1028]	'KYFQ' 'KSLQ']
>Contig_7_consensus_sequence_g71.t1 ; MatureChain: 19-336	2	[115 142]	['HCIS' 'RLLN']
		[65 167 644	['RYIT' 'KPFT'
>Contig_8_consensus_sequence_g92.t1 ; MatureChain: 20-2173	4	1925]	'RTFT' 'RCIT']
>Contig_8_consensus_sequence_g93.t1 ; MatureChain: 17-308	1	[81]	['HTYT']
			['KPLT' 'HLLI'
>Contig_17_consensus_sequence_g147.t1 ; MatureChain: 17-325	3	[3 142 266]	'KFFI']
			['KTYP' 'HELQ'
		[68 110 138 197	'RRFI' 'RTIT'
		214 249 265	'RPLE' 'KIYP'
>Contig_19_consensus_sequence_g156.t1 ; MatureChain: 21-359	8	302]	'RDIV' 'KLIH']
			['HDLD' 'RKLL'
			'KTLQ' 'KDFR'
			'RLLS' 'KDLD'
			'KDFG' 'KAIS'
			'RNFI' 'HFYM'
			'KAIQ' 'KAYG'
			'HLFS' 'RVIG'
		[26 32 84 97	'HDFD' 'RGML'
		112 123 224	'RIID' 'HVLS'
		242 332 364	'KLFY' 'RTFE'
		422 584 602	'KTLD' 'KTFT'
		634 762 778	'RSLN' 'HPLC'
		861 869 897	'RVIC' 'KTIK'
		956 967 1204	'RWYT' 'RTYC'
		1229 1236 1251	'HRFG' 'RGWR'
		1294 1301 1345	'KSLG' 'RGFQ'
		1367 1407 1473	'KWMI' 'KDLD'
		1487 1546 1566	'RLYE' 'RGIG'
		1573 1589 1617	'RDIL' 'HILQ'
		1637 1668 1689	'RIIN' 'KNWM'
		1695 1722	'HSLE' 'KKYC'
>Contig_23_consensus_sequence_g191.t1 ; MatureChain: 19-1836	43	1746]	'KNIE']
			['KDFC' 'KTFQ'
			'RELA' 'KIFI'
		[110 171 187	'RTFI' 'KSLI'
		200 231 235	'KFLS' 'KSWL'
		273 332 374	'RTFT' 'RKLG'
		413 479 566	'KQLQ' 'RTFC'
>Contig_23_consensus_sequence_g192.t1 ; MatureChain: 28-623	13	588]	'HCIP']
		[124 134 190	['KVWM' 'HIIH'
>Contig_24_consensus_sequence_g197.t1 ; MatureChain: 25-531	11	194 264 272	'HEIG' 'HTLG'

		313 406 442 466 473]	'HYFE' 'KDFS' 'RGIC' 'KKIT' 'KHYC' 'KPIS' 'RVFV']
>Contig_24_consensus_sequence_g199.t1 ; MatureChain: 21-413	7	[14 53 58 73 103 200 350]	['KYIV' 'RTFN' 'KNWN' 'KEIE' 'RALT' 'KVFQ' 'KVLE']
>Contig_27_consensus_sequence_g203.t1 ; MatureChain: 22-480	14	[0 41 144 169 177 206 231 237 302 321 341 348 421 439]	['HTLN' 'RKIF' 'RNIG' 'KQLL' 'RPLP' 'KELE' 'RNIL' 'RGLS' 'KELA' 'RILT' 'HGYQ' 'RYFF' 'KDLQ' 'RNLV']
>Contig_27_consensus_sequence_g209.t1 ; MatureChain: 20-340	12	[40 45 63 86 121 137 208 228 233 255 275 308]	['HSLT' 'RLLG' 'RGMG' 'KGIL' 'RGIG' 'HIWS' 'RAML' 'KTLR' 'KPLD' 'RTLW' 'HWFA' 'KWMD']
>Contig_29_consensus_sequence_g210.t1 ; MatureChain: 24-148	2	[15 61]	['RSLD' 'KMLH']
>Contig_30_consensus_sequence_g239.t1 ; MatureChain: 18-227	3	[34 52 115]	['RFYV' 'RRWT' 'KTIN']
>Contig_32_consensus_sequence_g247.t1 ; MatureChain: 19-1132	26	[5 47 62 193 246 262 270 330 379 406 471 494 556 603 699 755 793 816 825 886 900 937 1007 1013 1029 1095]	['HFMI' 'RSIP' 'KLLF' 'KNWL' 'KAYV' 'KGMP' 'RKLL' 'KIFF' 'KVLI' 'KGIT' 'KALD' 'KGLC' 'RGYC' 'RDYC' 'RSIA' 'HNFI' 'KFLQ' 'KMIT' 'KAFG' 'KFLK' 'RRLY' 'KAWT' 'RRFK' 'KGLD' 'RNMI' 'KWWR']
>Contig_39_consensus_sequence_g256.t1 ; MatureChain: 23-525	14	[35 49 82 170 211 233 241 275 347 391 437 441 461 483]	['KEFL' 'RFLP' 'RDIF' 'RNLG' 'KQLI' 'RGFQ' 'KSWD' 'HILD' 'RAIA' 'RYYF' 'RYMQ' 'HAWS' 'HSFR' 'KSID']
>Contig_40_consensus_sequence_g277.t1 ; MatureChain: 17-648	13	[16 66 106 167 173 217 264 313 352 408 542 555 587]	['RALP' 'KTFL' 'RALM' 'KVWR' 'RSIF' 'RALS' 'RVIS' 'RGFD' 'KAIE' 'HPLI' 'KEWP' 'RSWW' 'KSYE']
>Contig_47_consensus_sequence_g333.t1 ; MatureChain: 24-323	5	[21 28 38 52 77]	['KRYV' 'RDIP' 'KQML' 'KDLE' 'RSLW']
>Contig_60_consensus_sequence_g342.t1 ; MatureChain: 25-497	15	[11 15 81 86 100 127 251 255 265 310 345 410 422 459 469]	['RCLE' 'KSIN' 'HDLT' 'KALG' 'RNMK' 'RALH' 'KSLD' 'RRFS' 'KNIL' 'RLFK' 'RVFQ' 'RAIL' 'RYLN' 'KDIF' 'KPLH']
>Contig_60_consensus_sequence_g343.t1 ; MatureChain: 18-502	19	[1 20 58 77 130 161 181 215 225 269 273 287 318 331 359 385 408 430 438]	['KVLF' 'KVLR' 'KIIT' 'RTMR' 'HSYG' 'HRIP' 'KTID' 'KEYN' 'HFMG' 'RELN' 'HHIA' 'HGFP' 'RIWL' 'KALD' 'RALR' 'RNFL' 'HVMV' 'RALE' 'RFLE']
>Contig_60_consensus_sequence_g343.t2 ; MatureChain: 18-495	18	[1 20 58 77 130 161 181 218 262 266 280]	['KVLF' 'KVLR' 'KIIT' 'RTMR' 'HSYG' 'HRIP']

		311 324 352	'KTID' 'HFMG'
		378 401 423	'RELN' 'HHIA'
		431]	'HGFP' 'RIWL'
			'KALD' 'RALR'
			'RNFL' 'HVMV'
			'RALE' 'RFLE']
			['KKYI' 'RHYC'
			'KLFN' 'KTIN'
		[28 40 105 164	'KPFF' 'HLFL'
		177 225 312	'HRLQ' 'KDYN'
		422 436 476	'HELY' 'RPLL'
>Contig_67_consensus_sequence_g369.t1 ; MatureChain: 19-588	12	505 514]	'KVLH' 'KNLR']
			['KKYI' 'RHYC'
			'KLFN' 'KTIN'
		[28 40 105 164	'KPFF' 'HLFL'
		177 225 312	'HRLQ' 'KDYN'
		413 427 467	'HELY' 'RPLL'
>Contig_67_consensus_sequence_g369.t2 ; MatureChain: 19-579	12	496 505]	'KVLH' 'KNLR']
			['HIMR' 'KPYA'
			'KGIH' 'RVIA'
		[21 30 34 76	'RHIL' 'KVIE'
		135 217 291	'HKYV' 'KPLC'
		320 330 410	'RGFS' 'RWYN'
>Contig_67_consensus_sequence_g370.t1 ; MatureChain: 20-524	11	414]	'KNIR']
			['KLLS' 'RRMQ'
			'HQIA' 'HTWT'
		[10 153 160 165	'RNLF' 'KQLG'
		192 220 225	'HVIS' 'KDYE'
>Contig_66_consensus_sequence_g374.t1 ; MatureChain: 19-441	9	234 277]	'HNLT']
			['KSIP' 'RFIL'
		[242 378 454	'KSIY' 'KELE'
>Contig_57_consensus_sequence_g400.t1 ; MatureChain: 19-578	5	459 500]	'RGLS']
			['RVIS' 'RLYA'
			'KSIA' 'KKWE'
		[0 8 78 91 96	'HLMC' 'HTLR'
>Contig_57_consensus_sequence_g401.t1 ; MatureChain: 22-394	8	119 132 368]	'RTYA' 'HSWI']
		[96 185 217	['HFLA' 'RAFL'
>Contig_75_consensus_sequence_g411.t1 ; MatureChain: 20-408	4	233]	'KPLR' 'HLFP']
			['HSMY' 'RPLS'
		[264 281 479	'RELR' 'KPLA'
		485 497 507	'KPLG' 'HEIP'
>Contig_75_consensus_sequence_g413.t2 ; MatureChain: 23-560	7	534]	'RVYG']
			['RKLA' 'HDIT'
			'HHFG' 'KSFI'
			'KWIP' 'KWFK'
		[54 73 80 117	'KVFN' 'RAIG'
		141 192 205	'KNIW' 'KQWF'
		223 235 291	'HDIS' 'KGIR'
>Contig_83_consensus_sequence_g416.t1 ; MatureChain: 24-419	13	295 304 332]	'KKYQ']
			['RFWR' 'KDWI'
			'HDWA' 'RDLK'
			'KLLG' 'RWYP'
			'HKIV' 'KTIA'
			'KLLN' 'KLWG'
		[16 44 92 132	'HLYT' 'RLFD'
		156 196 277	'RELL' 'RTLL'
		289 295 300	'KVYI' 'RQLE'
		311 318 323	'RELG' 'HLIF'
		336 352 378	'KDWH' 'RSFI'
		384 431 435	'HDIP' 'KNLK'
		443 470 480	'KLIL' 'RVFK'
		553 611 619	'HPFV' 'HVFS'
>Contig_93_consensus_sequence_g440.t1 ; MatureChain: 26-896	28	693 743 854]	'KEWA' 'KGLK']
			['RIIY' 'RTFD'
		[39 45 108 208	'KSII' 'HYWA'
>Contig_92_consensus_sequence_g442.t1 ; MatureChain: 20-377	5	257]	'HAFE']
			['KKFC' 'KGYN'
		[110 177 196	'KSIP' 'KLYQ'
>Contig_92_consensus_sequence_g443.t1 ; MatureChain: 20-371	5	270 307]	'KDWL']
		[141 170 185	['RLLA' 'KGYT'
>Contig_92_consensus_sequence_g444.t1 ; MatureChain: 22-313	4	268]	'HLLL' 'KAIL']
			['RTLP' 'RQLR'
		[14 18 85 135	'RDWA' 'RGLI'
		162 175 186	'RAIA' 'RYIE'
		234 250 268	'KGLQ' 'KDYI'
>Contig_118_consensus_sequence_g463.t1 ; MatureChain: 19-431	12	321 391]	'KVIE' 'KILL'

			'KPLN' 'RGFT']
		[37 84 97 111 141 174 272 286 303 312 327 338 350 423 437 490 512 520 572 617 627 637 646 655 724 743 756 772 804]	['RCMG' 'RVIQ' 'RKIE' 'HVLE' 'KDIQ' 'KSLE' 'RFWA' 'KLFC' 'KFII' 'KRLL' 'HFFW' 'HTFK' 'KSIA' 'KILN' 'RDLL' 'KLYA' 'RYLS' 'KDWY' 'HALH' 'RQLS' 'RKLK' 'RFLH' 'KWLF' 'RLLE' 'RVLR' 'RLID' 'HSYG' 'KPFV' 'REIL']
>Contig_106_consensus_sequence_g470.t1 ; MatureChain: 22-886	29		
>Contig_106_consensus_sequence_g470.t2 ; MatureChain: 22-873	28	[37 84 97 111 141 174 272 286 303 312 327 338 350 423 477 499 507 559 604 614 624 633 642 711 730 743 759 791]	['RCMG' 'RVIQ' 'RKIE' 'HVLE' 'KDIQ' 'KSLE' 'RFWA' 'KLFC' 'KFII' 'KRLL' 'HFFW' 'HTFK' 'KSIA' 'KILN' 'KLYA' 'RYLS' 'KDWY' 'HALH' 'RQLS' 'RKLK' 'RFLH' 'KWLF' 'RLLE' 'RVLR' 'RLID' 'HSYG' 'KPFV' 'REIL']
>Contig_106_consensus_sequence_g473.t1 ; MatureChain: 21-117	2	[3 84]	['KTYS' 'HSLV']
>Contig_94_consensus_sequence_g480.t1 ; MatureChain: 19-626	5	[116 124 134 580 599]	['KDFI' 'HVWE' 'KDYY' 'KQFY' 'KTWA']
>Contig_94_consensus_sequence_g480.t2 ; MatureChain: 19-618	5	[108 116 126 572 591]	['KDFI' 'HVWE' 'KDYY' 'KQFY' 'KTWA']
>Contig_94_consensus_sequence_g481.t1 ; MatureChain: 34-1502	36	[3 83 98 160 183 273 300 322 370 427 458 476 498 502 618 643 651 694 698 709 747 829 845 924 1004 1058 1138 1179 1201 1222 1342 1347 1363 1400 1444 1451]	['RHLH' 'KTLG' 'HGFC' 'HLFV' 'HIYK' 'KELR' 'RHFN' 'HGIW' 'RSFE' 'RYMV' 'HGLM' 'KSIS' 'RIFP' 'KHML' 'RAIS' 'KVFD' 'RNLI' 'HRIK' 'KRFF' 'RTMS' 'KDYE' 'HTLN' 'HKFD' 'RHLF' 'KKFE' 'KDLQ' 'KPIK' 'RYWG' 'KAWV' 'HVFE' 'RLMA' 'KEMG' 'KKFC' 'KEFI' 'KNIS' 'KKIR']
>Contig_94_consensus_sequence_g481.t2 ; MatureChain: 34-1508 s	36	[3 83 98 160 183 273 300 322 370 427 458 476 498 502 618 643 651 694 698 709 747 829 845 924 1010 1064 1144 1185 1207 1228 1348 1353 1369 1406 1450 1457]	['RHLH' 'KTLG' 'HGFC' 'HLFV' 'HIYK' 'KELR' 'RHFN' 'HGIW' 'RSFE' 'RYMV' 'HGLM' 'KSIS' 'RIFP' 'KHML' 'RAIS' 'KVFD' 'RNLI' 'HRIK' 'KRFF' 'RTMS' 'KDYE' 'HTLN' 'HKFD' 'RHLF' 'KKFE' 'KDLQ' 'KPIK' 'RYWG' 'KAWV' 'HVFE' 'RLMA' 'KEMG' 'KKFC' 'KEFI' 'KNIS' 'KKIR']